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## THE ASSOCIATION OF THE DOUBLE BOND WITH THE LACTONE GROUP IN THE CARDIAC AGLUCONES.

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(Received for publication, June 30, 1926.)

By way of introduction a brief summary of the conclusions of a previous communication<sup>1</sup> may be given. Strophanthidin and all of its derivatives which possess both the lactone group and the double bond of the parent substance reduce Tollens' reagent and also give a positive nitroprusside reaction. The same has been found to be true of ouabain, gitoxin, and digitoxin. On saponification or hydrogenation these substances in general no longer give these reactions. On relactonization of the saponified substances a reaction will be again obtained where regeneration of the original substance is possible. In the case of strophanthidin the nature of this apparent association of the double bond with the lactone group appeared to be definitely established by the fact that the ethylal of oxidodianhydrostrophanthidin<sup>2</sup> yielded a keto acid on saponification which formed an oxime, thus characterizing it as a substituted crotonic lactone. It was therefore inferred that the unsaturated lactone group is present not only in strophanthidin but is in all likelihood a structural characteristic of all of the above "genins." The possible pharmacodynamic significance of this group was also considered in view of the fact that hydrogenation of the double bond had been shown to produce a marked effect upon the physiological behaviors of several of these substances.<sup>3</sup> It was necessary however to carry

<sup>1</sup> Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1925, lxvii, 333.

<sup>2</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxiv, 387.

<sup>3</sup> In the recent beautiful work of M. Cloetta (*Arch. exp. Path. u. Pharmacol.*, 1926, cxii, 261) this investigator has reached the same conclusion



the investigation beyond the point of mere color reactions in order to obtain if possible verification of such a conclusion by more direct methods. Even in the case of strophanthidin itself it became desirable to test the case more fully since a few observations had been made with several of its derivatives which did not seem to fit with the assumption that they are lactones of keto acids.<sup>4</sup>

As a first step and for confirmation of the preliminary observations made with the digitalis glucosides the study was extended to the aglucones themselves. Digitoxigenin<sup>5</sup> and gitoxigenin<sup>6</sup> like the parent glucosides were found to give strong positive nitroprusside reactions and when saponified this property was lost. In dilute pyridine solution they slowly reduced Tollens' solution. The hydrogenated substances<sup>7</sup> no longer gave the Legal test and their action upon Tollens' reagent was decidedly less marked, thus paralleling completely the experience with strophanthidin and ouabain. Like the latter substances the digitalis aglucones therefore exhibit towards these reagents a behavior unquestionably due to a similar if not identical association of the lactone group with the double bond.

regarding the effect of hydrogenation on "gitaligenin" and "bigitaligenin" (probably identical with gitoxigenin) thus adding to the observations of Windaus, Bohne, and Schwieger (*Ber. chem. Ges.*, 1924, lvii, 1388) on the effect of hydrogenation on digitalinum verum and those of Jacobs and Hoffmann<sup>1</sup> on ouabain.

<sup>4</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxiv, 386. In this article the case in point was the failure of the lactone acid obtained by oxidation of strophanthidin to give an oxime after saponification. This experiment is developed at greater length in the present communication. The formula for this acid previously given as  $C_{23}H_{30}O_7$  has since been changed to  $C_{23}H_{32}O_7$  (Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxv, 494).

<sup>5</sup> Cloetta, M., *Arch. exp. Path. u. Pharmacol.*, 1920, lxxxviii, 133.

<sup>6</sup> Windaus, A., and Schwarte, G., *Ber. chem. Ges.*, 1925, lviii, 1515.

<sup>7</sup> The material used in this test was obtained by hydrogenation of gitoxigenin with palladium black in acetic acid solution. The substance obtained formed needles which melted at 165–167°. The amount was too small for analysis. Dihydrodigitoxigenin was prepared in methyl alcoholic solution with colloidal palladium and formed delicate needles from dilute alcohol which melted at 110–113° and probably contained solvent of crystallization.

The attempt was next made to determine whether all of these substances would yield keto acids on saponification. In the case of strophanthidin the isomerizing effect of alkali compelled the choice of suitable derivatives, preferably not of the anhydrostrophanthidin series, the formation of which had apparently not involved the lactone group or the double bond of the parent substance. The lactone acid,  $C_{23}H_{32}O_7$ , obtained by the oxidation of the aldehyde group of strophanthidin to carboxyl, was employed. All attempts to prepare an oxime from this substance after gentle saponification resulted in recovery of the unchanged lactone acid. Analogous experiments were then made with pseudostrophanthidin in which both hydroxylamine and semicarbazide were employed with similar negative results and with recovery of unchanged material. Although in both of these substances just as in the case of the dianhydrostrophanthidin derivative the lactone group and the double bond were essential for the positive outcome of the nitroprusside reaction it appeared that hydroxy acids and not keto acids were produced by saponification. This was particularly suggested by the facility with which relactonization could be accomplished in contradistinction to the failure of such attempts with the ethylal of oxidodianhydrostrophanthidinic acid.

In order to complete our data a similar test was made with the ethylal of oxidoanhydrostrophanthidin<sup>8</sup> to see if this would parallel the experience with the dianhydro derivative. The acid obtained by its saponification proved indeed to be a keto acid which yielded an oxime without difficulty. Since this result could be obtained with substances only of the anhydrostrophanthidin series and which possessed at least one new double bond the possibility appeared that the appearance of this new double bond in the molecule may have caused a directed shift of the original olefinic linkage to a position  $\beta, \gamma$  within the lactone ring. That such a shift might have occurred with the participation of the alkali used for saponification was ruled out by the fact that when the new double bonds were removed by hydrogenation the crotonic lactone group could still be detected. For instance the previously described tetrahydrodianhydrodi-

<sup>8</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lix, 718.

lactone,  $C_{23}H_{30}O_4$ ,<sup>9</sup> on gentle saponification which involves only the lactone group in question readily yielded an oxime. Therefore if the lactone double bond occupies a position in the anhydro derivatives different from that in strophanthidin the shift must have occurred simultaneously with their formation. It is doubtful that alcoholic hydrochloric acid, the reagent used for their preparation, was responsible since strong aqueous acid was used in the preparation of pseudostrophanthidin. We shall return later to a discussion of the possible explanation of what has occurred.

Careful saponification experiments with ouabain showed that this substance readily lactonizes upon reacidification and in agreement with this result no oxime or semicarbazone could be obtained from the saponified lactone. Digitoxigenin was found to behave in identical manner. Unfortunately in the case of gitoxigenin a complication was encountered. This substance when once saponified by the most gentle treatment was found to suffer further alteration. Although relactonization occurred on acidification the resulting material was amorphous but still gave a nitroprusside reaction. In a parallel experiment in which an attempt was made to prepare an oxime a neutral nitrogen-free substance was recovered. With these substances therefore as in the case of the strophanthidin derivatives it has not been possible to prove directly the presence of the crotonic lactone group.

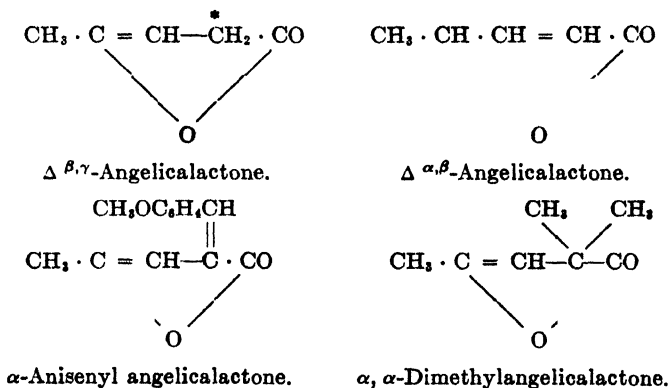
As a way out we have turned to a study of the angelicalactones and of certain of their derivatives. In his classical studies on the unsaturated lactones Thiele<sup>10</sup> described as a property characteristic of both the  $\Delta^{\alpha,\beta}$  and the  $\Delta^{\beta,\gamma}$  forms their immediate reducing action on alkaline silver solutions. Although the action of the cardiac aglucones on this reagent is much less marked and more gradual this might be explained by the complexity and the size of the molecule which might modify the speed of the reaction. Since no data were at hand with regard to the crotonic lactones and the nitroprusside test, we have investigated this point ourselves, and with very interesting and helpful results.

A very striking difference was at once observed in the behaviors

<sup>9</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxx, 503.

<sup>10</sup> Thiele, J., *Ann. Chem.*, 1901, cccix, 152.

of  $\Delta^{\alpha,\beta}$ - and  $\Delta^{\beta,\gamma}$ -angelicalactone.<sup>11</sup> The latter when treated with the reagent followed by a drop of alkali gave at once a deep red color which as usual gradually faded to a greenish yellow and on acidification changed to a deep violet. On the other hand the  $\Delta^{\alpha,\beta}$  form under the conditions employed developed a very faint color which slowly deepened and persisted much longer than in the case of the isomer. In this case the reaction was obviously due to gradual transformation into the  $\Delta^{\beta,\gamma}$ -lactone caused by the isomerizing effect of the alkali. The reaction could be explained as one between the reagent and 1 or 2 active hydrogen atoms of the  $\alpha$ -methylene group lying between the unsaturated carbonyl and the ethylene groups as follows:



In harmony with this view  $\alpha$ -anisenylangelicalactone<sup>12</sup> and  $\alpha, \alpha$ -dimethylangelicalactone<sup>13</sup> in which the active hydrogen atoms are replaced gave no reaction. The question as to whether both hydrogen atoms of the methylene group are necessary is now under investigation.<sup>14</sup>

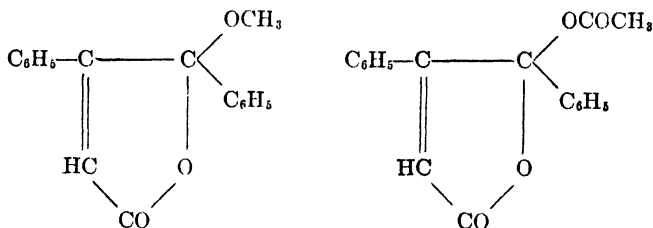
<sup>11</sup> Wolff, L., *Ann. Chem.*, 1885, cccxix, 249.

<sup>12</sup> Thiele, J., Tischbein, R., and Lossow, E., *Ann. Chem.*, 1901, cccxix, 185.

<sup>13</sup> Pinner, A., *Ber. chem. Ges.*, 1882, xv, 579.

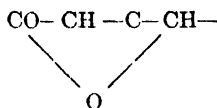
<sup>14</sup> It was of interest to determine whether the lactone ring itself was essential for the positive nitroprusside reaction since the cardiac aglucones, when once saponified, no longer give the reaction. In agreement with this observation  $\gamma$ -phenylisocrotonic acid,  $\text{C}_6\text{H}_5 \cdot \text{CH} = \text{CH} \cdot \text{CH}_2 \cdot \text{COOH}$ , gave no reaction, but its methyl ester gave the characteristic deep red color. This demonstrates that the lactone ring itself is not essential, but that the  $\alpha$ - $\text{CH}_2$  group becomes reactive when the carboxyl group is esterified or lactonized with simultaneous development of the unsaturated character of its CO group.

Thiele drew no distinction between the  $\Delta^{\alpha,\beta}$ - and the  $\Delta^{\beta,\gamma}$ -lactones in regard to their immediate reducing action on alkaline silver solutions but regarded this reaction as a common property of the two forms. We have reexamined this question however in view of the above observations and have found that if the aqueous solutions of the angelicalactones are chosen sufficiently dilute (0.2 per cent) a distinct difference in behavior is to be observed. Upon addition of a drop of Tollens' solution to the  $\Delta^{\beta,\gamma}$ -lactone the expected immediate precipitate of silver was obtained as stated by Thiele but the  $\Delta^{\alpha,\beta}$  form remained clear for at least 15 seconds and then gradually deposited silver. If instead of an alkaline solution an ammoniacal solution of silver was employed the difference in behavior was much more marked.  $\Delta^{\beta,\gamma}$ -angelicalactone gave an immediate silver precipitate while the isomer was without action upon it even on long standing. The action of alkaline silver solution upon the  $\Delta^{\alpha,\beta}$  form is therefore due to its preliminary transformation into the active  $\Delta^{\beta,\gamma}$  form and was due primarily to the active hydrogen of the  $\alpha$ -methylene group. In agreement with this conclusion  $\alpha$ -anisensylangelicalactone and  $\alpha,\alpha$ -dimethylangelicalactone do not reduce Tollens' reagent. Thiele<sup>10</sup> noted that the ability to reduce silver solutions appeared to depend upon the presence of a hydrogen atom on the  $\gamma$ -carbon atom since diphenylmethoxy- $\Delta^1$ -crotonic lactone and the acetate of diphenylhydroxy- $\Delta^1$ -crotonic lactone did not reduce, whereas both of the  $\beta,\gamma$ -diphenyl crotonic lactones reduced the reagent.



The proper interpretation of this observation shows that the presence of the  $\gamma$ -carbon hydrogen is necessary merely to make structurally possible the conversion of the  $\Delta^{\alpha,\beta}$  into the  $\Delta^{\beta,\gamma}$  form and does not itself react with the silver solution.

The outcome of the study of the behavior of the crotonic lactones towards Tollens' reagent and nitroprusside is in harmony with the view that the cardiac aglucones belong to this group of substances. The promptness of the appearance of the nitroprusside color would indicate that these substances are  $\Delta^{\beta,\gamma}$ -lactones while the gradual reduction of Tollens' reagent would suggest rather the  $\Delta^{\alpha,\beta}$  form. But any conclusion must be made to explain the failure of the experiments on oxime formation after saponification and the facility of relactonization in contrast with the behavior of the  $\gamma$ -keto acids which are the normal saponification products of the crotonic lactones. To explain this inconsistency two possibilities appear. The acids resulting after saponification may remain as  $\Delta^{\beta,\gamma}$ -hydroxy acids (the enolic form of the  $\gamma$ -keto acid) or perhaps as the  $\Delta^{\alpha,\beta}$ - $\gamma$ -hydroxy acid. The stability in such a case would have to be referred to obscure structural influences. As an alternative explanation the assumption may be made that the double bond is attached to the  $\beta$ -carbon atom but outside of the lactone ring as follows:



In this arrangement the  $\alpha$ -carbon atom lies between a carbonyl and an ethylene group. Its attached hydrogen should be active and exhibit the properties of a  $\Delta^{\beta,\gamma}$ -crotonic lactone with the exception that an unsaturated hydroxy acid would result on saponification. The formation of the true crotonic lactones of the anhydrostrophanthidin series could be attributed to the directive influence of newly formed double bonds in sufficiently close proximity to cause a shift of the original double bond to a position within the ring and apparently  $\beta,\gamma$ . The final solution of such a problem with substances of such complexity will be difficult. Although further investigations in this direction are being continued we have presented our observations in the present form because of the great importance which the association of the lactone group and the double bond have now assumed in the structural problem of the cardiac glucosides.

A further matter of interest has been the identical behavior shown by these substances on titration with bromine for double bonds by Winkler's method. Strophanthidin, ouabain, gitoxigenin, and digitoxigenin in spite of the presence of double bonds absorbed negligible amounts of bromine. On the other hand dianhydrostrophanthidin and its ethylal which possess two new double bonds add two mols of bromine.<sup>15</sup> The tetrahydrodianhydrodilactone<sup>9</sup>  $C_{23}H_{30}O_4$ , a derivative of dianhydrostrophanthidin in which these new double bonds have been hydrogenated, does not add bromine. Similarly digitaligenin which Windaus and Schwarte<sup>6</sup> have shown to be a dianhydrogitoxigenin was found to add two mols of bromine. In all of these substances therefore the lactone double bond does not add bromine. This is a property which also distinguishes the  $\Delta^{\alpha,\beta}$ - from the  $\Delta^{\beta,\gamma}$ -lactones and might perhaps support the assumption that these aglucones are of the former series. But it is also conceivable that very complex  $\Delta^{\beta,\gamma}$ -lactones might fail to add bromine, just as there are complex  $\Delta^{\alpha,\beta}$ -lactones which cannot be oxidized by permanganate to dihydroxy acids.<sup>16</sup> The above titrations can therefore be given only as an additional point in the resemblance of these substances.

#### EXPERIMENTAL.

##### *Attempts to Prepare Oximes after Saponification.*

Since the attempt to prepare oximes and semicarbazones from saponified pseudostrophanthidin, the lactone acid,  $C_{23}H_{32}O_7$ , ouabain, and digitoxigenin proved in each case to result in recovery of unchanged material, we shall give only the following examples

<sup>15</sup> In this connection trianhydrostrophanthidin (Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxi, 126) was found to have lost all tendency to add bromine possessed by dianhydrostrophanthidin. This is a most interesting confirmation of the previous experience (*loc. cit.*) of the behavior of this substance on hydrogenation. The new double bond as previously explained must participate in the formation of a conjugated system of three double bonds or a benzenoid structure.

<sup>16</sup>  $\Delta^1, \beta, \gamma$ -Diphenylcrotonic lactone in contrast to other  $\Delta^1$ -lactones was found by Thiele and Straus (*Ann. Chem.*, 1901, cccix, 156) to be affected by permanganate only with difficulty.

of our procedure. In the case of gitoxigenin it was found impossible to recover the substance unchanged in a preliminary saponification experiment due to the altering effect of the alkali.

0.5 gm. of pseudostrophanthidin was shaken in a machine in 20 cc. of alcohol and 10 cc. of 0.5 N sodium hydroxide until dissolved. The mixture was carefully titrated against phenolphthalein to neutrality which showed saponification to be complete. 0.3 gm. of hydroxylamine hydrochloride was dissolved in a small volume of water and carefully treated with sodium hydroxide solution until alkaline to phenolphthalein. This solution was then mixed with the saponified pseudostrophanthidin solution and was allowed to stand 24 hours, in some cases for a week at room temperature. The mixture was carefully acidified with acetic acid and allowed to evaporate slowly at ordinary temperature. A neutral substance slowly crystallized which was found to be recovered pseudostrophanthidin after recrystallization from dilute alcohol and possessed the required properties. In another experiment semicarbazide was used with similar results.

In the case of the lactone acid  $C_{23}H_{32}O_7$  (the oxidation product of strophanthidin) the reaction was carried on at water bath temperature but again unchanged starting material was obtained after reacidification.

In another experiment 0.1002 gm. of digitoxigenin was dissolved in a mixture of 8 cc. of 0.1 N NaOH and 8 cc. of alcohol and left 24 hours at room temperature. The solution no longer gave a nitroprusside reaction. When titrated back 2.33 cc. of 0.1 N NaOH had been consumed. Calculated 2.57 cc. A carefully neutralized solution of 0.2 gm. of hydroxylamine hydrochloride was added to the mixture and with a drop of dilute alkali the whole made faintly pink to phenolphthalein. In the course of several days the color deepened as if alkali had been set free due to relactonization. Long prismatic needles then separated from the solution which were neutral and nitrogen-free and gave a strong nitroprusside test. The melting point was 246–248° and the substance recovered was therefore digitoxigenin. The mother liquor on acidification with acetic acid and concentration at room temperature gave a second crop of neutral crystals which also proved to be somewhat less pure starting material.



*Anhydrostrophanthidin Derivatives.*

*Oxime of the Ethylal of Oxidoanhydrostrophanthidinic Acid.*—0.5 gm. of the ethylal of oxidoanhydrostrophanthidinic acid<sup>8</sup> was suspended in 5 cc. of 50 per cent alcohol and dissolved by careful addition of dilute sodium hydroxide solution. A concentrated solution of 0.3 gm. of hydroxylamine hydrochloride which was made alkaline to phenolphthalein was added and the mixture was left overnight at 20°. When acidified with acetic acid the oxime separated rapidly. Recrystallized from dilute alcohol it formed long thin lustrous leaflets which melted at 153–155°.

*Air-Dried Substance.* Dried at 100° and 15 mm. over H<sub>2</sub>SO<sub>4</sub>.

C<sub>26</sub>H<sub>37</sub>O<sub>6</sub>N·H<sub>2</sub>O. Calculated. H<sub>2</sub>O 3.87. Found. H<sub>2</sub>O 3.57.

*Anhydrous Substance.*

C<sub>25</sub>H<sub>37</sub>O<sub>6</sub>N. Calculated. C 67.07, H 8.34, N 3.13, OC<sub>2</sub>H<sub>5</sub> 10.06.

Found. " 66.75, " 8.26, " 3.18, " 8.24.

*Oxime of the Tetrahydrodianhydrolactone Acid.*—The tetrahydrodilactone was saponified as previously described<sup>9</sup> and carefully neutralized to phenolphthalein. On concentration *in vacuo* a lustrous crystalline deposit separated which was collected and proved to be a sodium salt. This was dissolved in alcohol diluted with an equal volume of water and acidified with acetic acid. The crystalline acid which immediately separated was collected with 25 per cent alcohol and was found to melt at about 200° after preliminary softening. However when this was dissolved in hot acetic acid and diluted carefully it crystallized again in a form which melted at 262° although with another sample recrystallized in a similar manner the melting point 275–276° was obtained.

0.3 gm. of this acid was suspended in 10 cc. of 50 per cent alcohol and brought into solution with a slight excess of alkali. To this an alkaline solution of 0.2 gm. of hydroxylamine hydrochloride was added and the mixture was left at 20° for 24 hours. On acidification with acetic acid the crystalline oxime slowly separated. Recrystallized from dilute alcohol it melted at 248–249°.

C<sub>23</sub>H<sub>33</sub>O<sub>5</sub>N. Calculated. C 68.44, H 8.25, N 3.48.

Found. " 68.62, " 8.46, " 3.41.

*Bromine Titrations.*

For the double bond titrations we have employed the method of Winkler.<sup>17</sup> The results obtained with the substances examined were as follows:

0.2104 gm. of strophanthidin used 0.10 cc. of 0.1 N KBrO<sub>3</sub> or a negligible amount.

0.1113 gm. of ethylal of oxidodianhydrostrophanthidin used 12.13 cc. of 0.1 N KBrO<sub>3</sub>. Calculated for 2Δs, 11.22 cc.

0.1011 gm. of dianhydrostrophanthidin used 10.81 cc. of 0.1 N KBrO<sub>3</sub>. Calculated for 2Δs, 10.98 cc.

0.0696 gm. of tetrahydrodianhydrodilactone, C<sub>23</sub>H<sub>30</sub>O<sub>4</sub>,<sup>9</sup> used no KBrO<sub>3</sub> solution.

0.1187 gm. of gitoxigenin used 0.62 cc. of 0.1 N KBrO<sub>3</sub> or a negligible amount.

0.1111 gm. of dianhydrogitoxigenin (digitaligenin) used 11.63 cc. of 0.1 N KBrO<sub>3</sub>. Calculated for 2Δs, 12.08 cc.

\* 0.0510 gm. of digitoxigenin used no KBrO<sub>3</sub> solution.

0.1219 gm. of ouabain used no KBrO<sub>3</sub> solution.

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<sup>17</sup> Meyer, H., *Analyse und Konstitutionsermittlung organischer Verbindungen*, Berlin, 4th edition, 1922, 1129.



# THE INFLUENCE OF INSULIN ON THE ACETALDEHYDE FORMATION IN THE BODY OF ANIMALS.

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Present knowledge seems to indicate that acetaldehyde is a necessary intermediary product of metabolism both in plants and animals. Its presence among the products of yeast fermentation of sugar is considered as evidence that it serves as the mother substance of alcohol, which is derived from it by reduction. Oxidation of acetaldehyde may also be accountable for the acetic acid which may occur as a product in the tissues of plants or in the excreta of animals. If conclusive evidence could be obtained that acetaldehyde does really occur in the tissues of animals it would be a distinct step forward, for not only would it indicate the manner of the metabolic breakdown of carbohydrates, but it would reveal a step in the intermediary processes by which the synthesis of fatty acids occurs out of carbohydrates and proteins. Thus acetaldehyde is well known readily to condense to aldol which by further condensation and oxidation may form fatty acids, or which may become partially oxidised so as to lead to the production of ketone bodies.

The evidence already exists for the presence of acetaldehyde in animal tissues. Thus Stepp (1921) detected a substance having the properties of acetaldehyde in diabetic urine. Neuberg (1923) and his coworkers have shown conclusively by their method of sulfate fixation, that acetaldehyde accumulates in considerable quantities when surviving tissues, such as liver or muscles, are incubated outside the body in the presence of suitable antiseptics. Hirsch (1923) found a substance which had the properties of acetaldehyde in the muscles of frogs. Stepp (1924) and his co-

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workers identified as acetaldehyde a volatile substance present in the urine and they showed that the urine of every normal human individual contains variable amounts of this substance. Fabre (1925) separated from large quantities of freshly drawn blood of horses a volatile substance which he proved was acetaldehyde, and he has also shown that the blood of the depancreatized dogs contains 10 to 20 times more of this substance than the blood of a normal dog.

Since insulin has a rapid and more marked influence on the metabolism of sugar in the animal, it is natural to enquire whether it may not lead to a change in the acetaldehyde content of the tissues. Neuberg and Gottschalk (1923) have shown that this is at least the case in incubated suspensions of liver and muscle when the aldehyde is fixed by sulfite as soon as it is formed and the first experiments of the present investigation confirm their findings. This does not necessarily mean, however, that acetaldehyde will be formed in the intact animal, and the main part of the present work is concerned with this problem.

#### *Determination of Acetaldehyde.*

The determination of acetaldehyde in the tissues is surrounded by many difficulties since it is both highly volatile and a very unstable substance which readily oxidises or polymerises. The material for analysis must be collected at low temperatures with precautions against loss of acetaldehyde by volatilization. Most of the present determinations were made on blood which was collected as quickly as possible in chilled vessels, in which it was mixed at once with the Folin-Wu reagents for proteins. The precipitate of proteins was separated by means of the centrifuge, and the free acetaldehyde was determined in the supernatant fluid by the method of Fabre, which consists in keeping the filtrate at 38°C. for 2 days, in the presence of Nessler reagent suspended over it in a flat dish. After this treatment the solution was transferred to a Clausen flask and distilled at a temperature of about 105–110°C. with nitrogen bubbling through it, the distillate being collected in Nessler reagent.

The method of Bougault and Gros (1922) is suitable for the determination of very small quantities of acetaldehyde, and it gives satisfactory results.

The Nessler reagent oxidises acetaldehyde to acetic acid and the equivalent of metallic mercury is separated from the solution. This mercury can be easily determined iodometrically, by neutralising the solution with HCl and mixing with 0.1 N I solution. The iodine combines with mercury to form mercuric iodide and the iodine left uncombined can be determined by N/200  $\text{Na}_2\text{S}_2\text{O}_3$ . 1 cc. of N/200  $\text{Na}_2\text{S}_2\text{O}_3$  equals 0.11 mg. of  $\text{CH}_3\text{COH}$ .

To test the reliability of the method a standard solution of acetaldehyde, 5 mg. per 100 cc., was used. The results are given in Table I.

For determination of acetaldehyde in urine, the urine after the addition of acid is distilled in a current of nitrogen, and acet-

TABLE I.  
*Reliability of Method.*

	100 cc. of the standard solution.		100 cc. of standard solution mixed with 20 gm. of rabbit liver.
	Without distillation.	In distillate.	In distillate.
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Bougault and Gros method. ....	4.97	4.95	4.88
Brochet and Cambier (1895) method. ....	4.95	4.85	4.81

aldehyde is determined in the distillate by the Bougault-Gros (1922) method. The acidification is necessary to prevent the distillation of ammonia and the volatile amines.

In the solid tissues total acetaldehyde only was determined. The organs, after the removal from the animals, were frozen quickly in liquid air and either pulverised or crushed in a mortar or passed through a chilled sausage machine; the pulp was then mixed with an equal quantity of 0.5 per cent oxalic acid solution and transferred to a distilling flask, acetaldehyde being determined in the distillate by the Bougault-Gros (1922) method.

#### *Formation of Acetaldehyde in Tissues in Vitro.*

In these experiments the original method of Gottschalk and Neuberg was used, and the observations were extended to in-

TABLE II.  
*Influence of Insulin on Acetaldehyde Formation in Excised Tissues.*

Tissue.	Tyrode solution with:		Time of incubation. hrs.	Insulin. cc.		CH <sub>3</sub> COH mg. per 1000 cc.
Guinea pig liver, 10 gm.	50 cc. glucose 1 per cent.	50 " " 1 " "	16	2 + 2 after 2 hrs.		145.2
" 10 "	50 " " 1 " "	50 " " 1 " "		None.		96.8
Rabbit liver, 40 "	100 cc. fructose 1 per cent.	100 " " 1 " "	6	2 + 2 after 2 hrs.		7.7
" 40 "	1000 " " 1 " "	1000 " " 1 " "		None.		3.3
Dog " 50 "	100 " " 1 " "	100 " " 1 " "	6	2 + 2 after 2 hrs.		18.6
" 50 "	100 " " 1 " "	100 " " 1 " "		None.		9.3
Cat " 25 "	50 " " 1 " "	50 " " 1 " "	6	2 + 2 after 2 hrs.		9.4
" 25 "	50 " " 1 " "	50 " " 1 " "		None.		0.88
Catfish " 10 "	50 " " 1 " "	50 " " 1 " "	8	2 + 2 after 3 hrs.		4.5
" 10 "	50 " " 1 " "	50 " " 1 " "		None.		1.7
Rabbit muscle, 50 "	100 " glucose 1 " "	100 " " 1 " "	16	2 + 2 after 6 hrs.		146.0
" 50 "	100 " " 1 " "	100 " " 1 " "		None.		114.4
Catfish " 25 "	100 " fructose 1 " "	100 " " 1 " "	7	2 + 2 after 3 hrs.		34.0
" 25 "	50 " " 1 " "	50 " " 1 " "		None.		9.0

clude the muscles of the rabbit and catfish, and the liver of the dog, cat, catfish, and rabbit.

TABLE III.  
*Influence of Pituitrin on Acetaldehyde Formation in Excised Tissues.*

Tissue.		Tyrode solution with:	Time of incubation.	Pituitrin.	CH <sub>3</sub> COH
			hrs.	cc.	mg. per 1000 cc.
Cat liver,	25 gm.	50 cc. fructose 1 per cent	12	2	77.5
	25 "	50 cc. fructose 1 per cent		None.	61.8
Rabbit liver,	25 "	50 cc. fructose 1 per cent.	6	2	30.0
	20 "	50 cc. fructose 1 per cent.		None.	31.0

TABLE IV.  
*Formation of Acetaldehyde in the Rabbit.*

	Rabbit 1. Normal.	Rabbit 2. Glucose injected.	Rabbit 3. Insulin injected.	Rabbit 4. Fasted. Insulin injected.	Rabbit 5. Fructose injected.	Rabbit 6. Fructose and insulin injected.	Rabbit 7. Glucose and insulin injected.	
	mg. per 1000 gm.	mg. per 1000 gm.	mg. per 1000 gm.	mg. per 1000 gm.	mg. per 1000 gm.	mg. per 1000 gm.	Experiment 1.	Experiment 2.
Blood,								
Free acetaldehyde .....	0.54							
Total acetaldehyde .....	0.99	1.34	Trace.	Trace.	1.54	3.08	1.1	1.45
Sugar.....		0.25			0.44	0.13	0.11	0.45
Liver, total acetaldehyde.	3.14	2.1	3.81	2.54	3.21	4.74	9.31	4.4
Muscles, free acetaldehyde	1.22	1.71	1.91	1.02	1.91	6.96	1.91	3.21

The influence on acetaldehyde formation of glucose and fructose as well as of insulin was studied. To prevent bacteria grow-



ing during the incubation at 38°C. about 0.08 per cent of optochin was added to the organ pulp mixture.

In every case insulin caused an increase in the formation of acetaldehyde and this was more pronounced in the presence of fructose than with glucose, a result which agrees with those of Gottschalk and Neuberg (1923). Pituitrin (Lilly) was not found to affect acetaldehyde formation (Table III).

#### *Formation of Acetaldehyde in Tissues in Vivo.*

For the exact determination of acetaldehyde in normal blood about 250 to 500 gm. are necessary. It is possible, however, by using a more dilute thiosulfate solution—N/400—to determine acetaldehyde approximately in 25 cc. of blood when it is present in a concentration of about 2 mg. per kilo. The difficulties in obtaining a sufficient quantity of blood, or of tissues, from small animals forced us in many cases to use 25 gm. quantities for the analyses, and in these cases the results can be considered as only of approximate accuracy.

#### *Formation of Acetaldehyde in the Body of the Rabbit.*

1. *Amount Normally Present.*—Six normal rabbits were killed and 250 gm. of blood, 350 gm. of liver, and 1000 gm. of muscles were obtained. The quantities of acetaldehyde in these tissues are given in Column 1 of Table IV.

2. *Influence of Glucose.*—A rabbit weighing 1850 gm. received an injection of 10 cc. of 20 per cent glucose solution, subcutaneously, and after half an hour a second injection of the same amount was given intravenously. In half an hour after the second injection the animal was killed, and 38 gm. of blood, 80 gm. of liver, and 180 gm. of muscles, were used for the determination of acetaldehyde (Column 2, Table IV).

3. *Influence of Insulin.*—Each of two rabbits received 3 units of insulin 45 minutes before being killed. Quantities of 70 gm. of blood, 150 gm. of liver, and 300 gm. of muscles were used for determination of acetaldehyde (Column 3, Table IV). In another experiment insulin was injected into two rabbits from which food had been withheld for 3 days, and 65 gm. of blood, 140 gm. of liver, and 250 gm. of muscles were used for analysis (Column 4, Table IV).

4. *Influence of Fructose.*—A rabbit weighing 2000 gm. received, 45 minutes before death, 16 cc. of 50 per cent fructose solution subcutaneously, and 45 gm. of blood, 65 gm. of liver, and 130 gm. of muscles were used for analysis (Column 5, Table IV).

5. *Influence of Fructose and Insulin.*—A rabbit weighing 1925 gm. was used for the determination of acetaldehyde in 35 gm. of blood, 45 gm. of liver, and 130 gm. of muscles after the subcutaneous injection of 16 cc. of 50 per cent fructose solution and 3 units of insulin. The rabbit was killed 45 minutes after the injection (Column 6, Table IV).

6. *Influence of Insulin and Glucose.*—(1) A rabbit weighing 1980 gm. received at intervals of half an hour three injections of 10 cc. of 20 per cent glucose solution and 3 units of insulin subcutaneously. It was killed 45 minutes after the last injection, and determination of acetaldehyde was carried out on 50 gm. of blood, 38 gm. of liver, and 175 gm. of muscles (Column 7, Table IV). (2) A similar observation was carried out on a second rabbit, weighing 3150 gm.

Table IV shows that insulin, when given with either glucose or fructose, decidedly increased the quantity of acetaldehyde in the liver and muscles, and, in the case of fructose, in the blood. Both glucose and fructose given alone also seem to increase slightly the quantity of acetaldehyde in the blood, but we place no emphasis on this result because of the relatively small quantities of the blood available for the determination.

*Influence of Alcohol Followed by Insulin on Blood Acetaldehyde.*

Special attention was paid to the effect of ethyl alcohol. Two rabbits (A and B) received subcutaneously 2.5 gm. per kilo of alcohol, and one of them 1 unit of insulin. Two other rabbits (C and D) received 5 cc. of 50 per cent alcohol per kilo by stomach tube, and one of them 1 unit of insulin subcutaneously. After 45 minutes 50 cc. of blood were taken from the heart for the determination of acetaldehyde, sugar, and alcohol (Widmark method, 1922). The results are given in Table V.

There is no doubt that alcohol causes an increase of acetaldehyde of the blood and this becomes about 100 per cent more marked after the injection of insulin.

In another experiment alcohol and sugar only were determined

in the blood. Two rabbits (Nos. 1 and 2) of 2000 gm. each were used, and each was injected with 0.25 gm. per kilo of alcohol subcutaneously, and after 45 minutes, samples of 28 cc. of heart blood were taken for the determination. On the following day the same rabbits received the same quantities of alcohol along with 1 unit of insulin. After 45 minutes the bloods for analysis were taken. The results are shown in Table VI.

Insulin brings about a decrease of alcohol when small quantities of alcohol are present in blood.

TABLE V.  
*Influence of Alcohol Followed by Insulin on Blood Acetaldehyde.*

	2.5 gm. of alcohol per kilo subcutaneously.		5 cc. of 50 per cent alcohol by mouth.	
	Rabbit A.	Rabbit B. 1 unit of insulin.	Rabbit C.	Rabbit D. 1 unit of insulin.
Sugar, per cent.....	0.11	1.09	0.135	0.06
Alcohol, per cent.....	0.753	0.458	0.254	0.350
Acetaldehyde, mg. per 1000 gm.....	15.4	34.65	7.8	13.2

TABLE VI.  
*Influence of Alcohol Followed by Insulin on Blood Sugar.*

	Rabbit 1.		Rabbit 2.	
		Insulin.		Insulin.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Sugar.....	0.119	0.059	0.092	0.051
Alcohol.....	0.164	0.111	0.098	0.052

### *Acetaldehyde Formation in Dogs.*

The observations are divided into three groups: (a) normal dog, (b) a dog injected with insulin and sugar, (c) depancreatized dogs. (a) 642 gm. of blood, 560 gm. of liver, and 448 gm. of muscles were taken immediately after death by stunning, from a normal dog weighing 25 kilos. (b) A dog weighing 21 kilos, was injected subcutaneously with 2.8 cc. of insulin and 60 cc. of 50 per cent glucose solution. 2 hours later the animal was in-

jected again with 2 cc. of insulin and 40 cc. of 20 per cent glucose solution. 45 minutes later the dog was killed and 185 gm. of blood, 234 gm. of liver, and 429 gm. of muscles were used for the determination of acetaldehyde. (c) Sufficient blood (200 cc.) was removed from three depancreatized dogs as follows: Dog 1 depancreatized 3 months previously and kept without insulin for 3 days. Dog 2 depancreatized 7 days previously. Dog 3 depancreatized 3 days previously, received 0.4 cc. of insulin 24 hours before the experiment. The results are given in Table VII.

The injection of glucose and insulin caused an increase in the acetaldehyde content of blood and muscles in the normal animal,

TABLE VII.  
*Acetaldehyde Formation in Dogs.*

Dog.	Normal.	Insulin and glucose injected.	Depancreatized dogs.		
			Dog 1.	Dog 2.	Dog 3. (insulin injected).
	gm. per 1000 gm.	gm. per 1000 gm.	gm. per 1000 gm.	gm. per 1000 gm.	gm. per 1000 gm.
Blood.					
Free acetaldehyde.....	0.42	2.13	3.17	1.25	0.31
Total acetaldehyde.....	0.85	4.8	4.12	1.73	0.54
Sugar.....		0.092	0.23	0.19	0.14
Liver, total acetaldehyde.....	3.44	3.6			
Muscles, total acetaldehyde.....	0.94	2.7			

and there was also an excess in the blood of the depancreatized dogs. This was reduced to normal by insulin. An increase in acetaldehyde was also observed by examination of urine collected by catheter from a depancreatized dog (acetaldehyde 18.0 mg. per liter), from which food had been withheld for 24 hours. The same dog after the injection of half a unit of insulin, excreted urine with 1.1 mg. of acetaldehyde per liter. The urine of a normal fasted dog contained 1.61 mg. per liter of acetaldehyde.

TABLE VIII.

*Acetaldehyde of the Blood of the Dog after Alcohol and Insulin.*

Experiment No.		After:				
		1 hr.	2 hrs.	3 hrs.	4 hrs.	
		mg. per 1000 gm.	mg. per 1000 gm.	mg. per 1000 gm.	mg. per 1000 gm.	mg. per 1000 gm.
1	Sugar.	0.072				
	Alcohol.	0.768				
	Acetaldehyde.	7.54				
2	Sugar.	0.09				
	Alcohol.	0.283				
	Acetaldehyde.	11.52				
Insulin injected.						
3	Sugar.	0.065	0.045	0.046	0.054	
	Alcohol.	0.0283	0.429	0.532	0.299	
	Acetaldehyde.	18.8	24.3	7.5	10.3	
4	Sugar.	0.114	0.105	0.083		
	Alcohol.	0.870	0.729	0.429		
	Acetaldehyde.	7.35	7.60	7.30		
5	Sugar.	0.066	0.064	0.061	0.059	
	Alcohol.	0.345	0.345	0.161	0.051	0.041 without alco- hol.
6	Sugar.	0.093	0.093	0.083	0.082	
	Alcohol.	0.418	0.559	0.469	0.220	0.087 without alco- hol.
7	Sugar.	0.059	0.043	0.054	0.045	
	Alcohol.	0.033				0.043 without alco- hol.
8	Sugar.	0.085	0.085			
	Alcohol.	0.051				0.084 without alco- hol.

*Influence of Alcohol and Insulin on the Concentration of Acetaldehyde in the Blood of the Dog.*

1. A dog (10 kilos) received 50 cc. of 50 per cent alcohol by stomach tube, and after 1 hour 28 cc. of blood were removed for analysis.

2. The same dog 4 days later received the same quantity of alcohol along with 1 cc. of insulin subcutaneously, and after 1 hour a sample of blood was removed for analysis.

3. A dog (20 kilos) received 100 cc. of 50 per cent alcohol by stomach tube and 2 cc. of insulin subcutaneously, and at intervals of 1 hour each four samples of the blood (28 cc. each), were removed for analysis.

4. 4 days later the preceding observation was repeated on the same animal.

5. A dog (17 kilos) received 50 cc. of alcohol (50 per cent) and 2 cc. of insulin subcutaneously, and at intervals of 1 hour each four samples of blood were removed for analysis.

6. A day later the preceding experiment was repeated on the same animal.

7. A dog (7 kilos) received 2.5 cc. of alcohol subcutaneously and 2 cc. of insulin. In intervals of 1 hour each four samples of blood were removed for analysis.

8. A day later the preceding experiment was repeated on the same animal.

Alcohol alone increases the acetaldehyde of the blood and there is some evidence that when injected in large doses the blood sugar may be slightly raised, but this is doubtful. Insulin injected along with alcohol causes the increase of acetaldehyde to be more pronounced. At the same time the alcohol concentration of the blood is reduced below the level observed in the same animal injected with alcohol alone.

*Action of Insulin on Blood Acetaldehyde after the Injection of  $\text{CH}_3\text{COH}$  in Rabbits.*

Each of four rabbits received *per os* about 1 cc. per kilo of acetaldehyde and at intervals of  $\frac{1}{2}$  to 4 hours samples of the blood of 3 to 5 cc. were taken for determination of sugar and acetaldehyde. Two of the rabbits (Nos. 5 and 6) also received sub-

cutaneously 1.5 cc. of acetaldehyde per kilo and one of them (No. 6), also received 3 units of insulin. Samples of blood were taken for analysis at intervals of  $\frac{1}{2}$  to 2 hours. The results are given in Table IX.

TABLE IX.

*Effect of Injection of Acetaldehyde on Acetaldehyde and Sugar of Rabbits.*

Acetaldehyde given per os.					Acetaldehyde given subcutaneously.			
hrs.	Rabbit 1. No insulin.	Rabbit 2. Insulin 1 unit.	Rabbit 3. No insulin.	Rabbit 4. Insulin 3 units.	Rabbit 5.		Rabbit 6.	
	mg. per 1000 cc.	mg. per 1000 cc.	mg. per 1000 cc.	mg. per 1000 cc.	No insulin.		Insulin.	
						Sugar.		Sugar.
$\frac{1}{2}$	150	37	83	106.3	107.5	0.232	152	0.167
1	126	43	64			0.230		
2	40		50	19.5	55	0.230	73	0.107
3								
4	33	00	33	17.0	18.3	0.292	55	0.109
6	90	00	23	00	00			
8			00					

TABLE X.

*Effect of Acetaldehyde on the Acetaldehyde and Sugar of the Blood of the Dog.*

hrs.	Without insulin.		With insulin.	
	Sugar.	Acetaldehyde.	Sugar.	Acetaldehyde.
	per cent.	mg. per 1000 cc.	per cent.	mg. per 1000 cc.
1	0.085	81.4	0.063	28.0
2	0.094	66.2	0.056	33.0
3	0.059	24.2	0.040	15.0

A dog (10 kilos) received 5 cc. of acetaldehyde subcutaneously, and during the following 4 hours three samples of blood were taken at intervals of 1 to 2 hours. A day later the same dog received subcutaneously 5 cc. of acetaldehyde and 3 cc. of insulin, and three samples of blood were taken as above. The results are shown in Table X.

*Fate of Acetaldehyde in the Body.*

A rabbit received 1 cc. of acetaldehyde per kilo subcutaneously, and after half an hour four samples of the blood were taken (5 cc. each), two from the right and two from the left heart. The venous blood contained 194 to 190 mg. per kilo of acetaldehyde, the arterial blood 172.5 to 180 mg. per kilo. The small differences in the concentration between the two is to be accounted for by excretion by the lungs. In order to measure the extent of this, a rabbit received subcutaneously 1 cc. of acetaldehyde per 2 kilos, and was placed in a respiratory chamber connected with two wash bottles in series each containing 40 cc. of Nessler's reagent. Through the chamber and the wash bottles a slow current of air was aspirated by means of a water pump. During 6 hours 1.166 mg. of acetaldehyde collected in the Nessler reagent and 2.1 mg. were recovered in the urine.

A second rabbit received 1 cc. per kilo of acetaldehyde and excreted by the lungs during the 1st hour 2.31 mg., during the 2nd hour 1.45 mg., during the 3rd hour 0.62 mg., and during the 4th hour 0.00 mg. of acetaldehyde. The urine contained 5.67 mg. These results indicate that acetaldehyde is excreted in very small amounts through the lungs and urine, most of it being metabolised in the organism.

*Identification of Liver Acetaldehyde.*

For this purpose 260 gm. of rabbit liver were distilled in a slow current of nitrogen at a temperature of 100° and about 15 cc. of distillate were obtained. This was mixed with 0.1 gm. of dimedon dissolved in 1 cc. of pure alcohol and with 0.3 gm. of NaCl. After 24 hours a white crystalline precipitate separated out, which was filtered, washed with water, and recrystallised from 0.5 cc. of hot 85 per cent alcohol. About 7 mg. of white crystals were obtained, which had the same crystalline structure under the microscope as pure aldomedon. M.p. of crystals, 140°. The melting point of crystals mixed with pure aldomedon was 140-141°.

## DISCUSSION.

The observation of Neuberg and Gottschalk is confirmed, that insulin accelerates the formation of acetaldehyde in the tissues



of animals incubated outside the body in the presence of calcium sulfite.

The injection of the large doses of insulin along with sugar into normal animals usually results in an increase in the acetaldehyde of the tissues and blood, but inasmuch as the acetaldehyde present in these must represent a balance between its formation and decomposition the changes may be insignificant.

In the blood and probably also in the organs, acetaldehyde exists partly in a free state and partly in the form of some unknown compound which yields acetaldehyde on heating to  $100^{\circ}$ , but the quantities appear, from the approximate methods available for determination, to vary within fairly wide limits. Injection of sugar alone or possible intermediary products of carbohydrate metabolism seems to increase the acetaldehyde of the blood. Both the blood and the urine of depancreatised dogs contain more acetaldehyde than that of the normal animal, and insulin can restore the amounts to the normal level. This hyperacetaldermia in diabetes may have a significant connection with ketosis and lipemia, or with glyconeogenesis out of protein and fat.

Acetaldehyde becomes decidedly increased in the blood after the administration of alcohol. The injection of insulin increases this hyperacetaldermia and causes the alcohol to disappear more quickly from the blood. There is some indication also that the alcohol retards the fall in blood sugar following the injection of insulin. Insulin, therefore, seems to accelerate the oxidation of alcohol to acetaldehyde in the animal body.

After the injection of moderate quantities of acetaldehyde, traces only are excreted in the expired air and urine. The curve of its concentration in the blood after the injection of  $\text{CH}_3\text{COH}$  falls rapidly to a level of about 20 mg. per kilo, where it may persist for several hours. As it disappears from the blood most of it is probably converted into ethyl alcohol and acetic acid which are oxidised to  $\text{CO}_2$ , though some of it may undergo condensation and so lead to a synthesis of fatty acids or carbohydrates. Insulin appears to accelerate somewhat the rate of disappearance of acetaldehyde from the blood. The fact that the blood sugar becomes raised after the injection of aldehyde may have no other significance than that it has excited glycogenolysis (of Sansum and Woodyatt, etc.).

## CONCLUSIONS.

1. Insulin increases the formation of acetaldehyde in the organs *in vitro*.

2. The injection into normal animals of insulin along with sugar increases acetaldehyde in the organs.

3. An excess of acetaldehyde is present in the blood and urine of depancreatized animals and insulin restores it to the normal level.

4. Excess of acetaldehyde appears in the blood following the administration of ethyl alcohol, and, when insulin is also given, it accentuates this increase, but at the same time accelerates the return to the normal level.

5. After the injection of moderate quantities of acetaldehyde only small amounts are excreted through the kidneys and lungs, and insulin seems to accelerate disappearance of blood acetaldehyde under these conditions.

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## INTESTINAL CHEMISTRY.

### IV. A METHOD FOR THE STUDY OF FOOD UTILIZATION OR DIGESTIBILITY.

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College of Medicine, Chicago.)

(Received for publication, June 28, 1926.)

We mean by the utilization of a food substance the difference between the amount of this substance present in the diet and the amount excreted in the feces. In the case of proteins, fats, and complex carbohydrates utilization depends chiefly on and is a measure of the digestibility of the food substance. In the case of inorganic salts and simple sugars which do not require digestion what is really measured is absorbability. These measurements of the proportions of the ingested food substances made available to the uses of the body by digestion and absorption are necessary to the estimation of the nutritive value of foods. They serve also as indices of the digestive and absorptive powers of the gastrointestinal tract in health and in disease, and of the factors influencing digestion and absorption.

In spite of the many practical considerations involved, however, utilization studies are carried out with comparative infrequency in investigational work and almost never for clinical purposes. For example, hundreds of researches have been carried out in rickets in which disease calcium absorption is believed to be of fundamental importance, yet investigators prefer to use as measures of calcium absorption the rates of growth of animals or the formation of lines of calcification in the bones. Such methods are valuable but they are not quantitative measures of calcium absorbed. Studies of the digestion of other food substances by the smaller animals commonly used in investigational work are comparatively rare. In experiments on men also utilization experiments appear to be avoided wherever possible. The chief reason

that such determinations are not more frequently employed is that they take so much time to carry out. The purpose of the present paper is to present a simplified procedure which it is hoped may extend the usefulness of this type of determination.

In the standard method a carmine capsule is given at breakfast of the 1st day of the experiment and a uniform diet is then fed for a period of 5 to 7 days, another carmine capsule being given at the last meal of the period. Feces are collected and that lying between the two carmine markers and including that colored with the first carmine are kept at a freezing temperature until the end of the experiment. Then all feces are mixed and portions weighed out for analysis. In certain cases as in the determination of fat utilization each stool must be separately analyzed because of alterations in the fat content on standing for even a few days. The diet must consist of weighed amounts of foods and these foods must be analyzed. Periods of less than 5 days have not generally proven practicable because of the difficulty in obtaining a satisfactory separation of the feces of the experimental diet. Carmine may become mixed with the diets of preceding and following days and in certain cases especially where the feces form scybala or are of a very soft consistency the error thus introduced may be considerable.

In making utilization experiments on small animals, such as albino rats which are in many cases the most convenient animals to employ, additional difficulties have greatly limited the use of the usual methods. In the first place food may be scattered to a greater or less extent depending on the nature of the diet and may be lost or contaminated with urine, feces, or water, and moist foods may lose moisture so that ordinary precautions will not always insure an accurate estimate of food ingested. The feces may furthermore become in part admixed with urine or with food and as the latter may be ten or twenty times as rich in nitrogen, phosphorus, etc., as the feces and as in this method a complete collection of feces must be attempted, contamination may materially affect the results. As a result of the difficulties encountered only a few utilization experiments on rats have been recorded.

The proposed method consists in the addition to the food of

small amounts of iron oxide (or other suitable substance). This being practically insoluble and unabsorbable and of suitable physical consistency follows the food in the course of its digestion through the intestine and is excreted with practical completeness in the feces. The amount of iron in the food and in the feces is readily determined and the ratios of the amount of iron to the amount of any food substance in the diet and in the feces determined and the percentage utilization calculated. For example if the ratio of glucose to iron in the food is 100:1 and in the feces 1:1, divide the fecal ratio by the food ratio ( $1 \div 100$ ) and obtain 0.01, or 1 per cent of glucose as not utilized and 99 per cent as the utilization of this sugar.

In the simplest cases utilization may be thus determined by putting in a cage with one or more animals a weighed amount of food and after a day or two collecting a few uncontaminated specimens of feces. The ratios of, for example, glucose to iron are determined for food and feces, and percentage utilization is calculated. If more than one animal is used an average utilization value for the group is obtained from a single determination. The foods and feces need not even be weighed for analysis, if, as in the case of glucose or calcium for example, both iron and the food substance can readily be determined in the same portion, because we are concerned primarily with ratios and not with absolute amounts. Thus only an approximate estimate need ordinarily be made of the amount of food ingested. Separation and complete collection of feces are unnecessary. Instead of minute attention no special care of animals is necessary. Groups may be run as well as individuals. The iron determination may be easily carried out after ashing or direct extraction with hydrochloric acid. This simplest form of procedure may be elaborated by attempting estimation of exact amounts of food ingested, total collection of feces, and in other ways up to the point where it becomes a practical duplication or check on the usual procedure. For a great many purposes, however, these extra precautions are quite unnecessary to the obtaining of the desired results. The procedure suggested is thus much easier to carry out than the usual method and becomes available in many cases where the more elaborate method would not be employed. Furthermore, the

fact that single specimens of feces may be used for determinations makes possible certain types of experiment which could not otherwise be made.

The extent to which this procedure may replace the usual method can only be demonstrated by experiments carried out under a variety of conditions. The more elaborate procedure carefully carried out may give more accurate results in certain cases as where a variety of foods are being given and metabolic balances are being determined, but in many instances the slight increase in accuracy will not justify the greater expenditure of time.

Iron oxide or hydroxide is a useful key substance to employ because it is easily determined in foods and feces, is not appreciably absorbed from the intestines, can be readily incorporated with foods, and because, on account of its insolubility, it has little pharmacological action.

In determining the percentage utilization of food substances which are quite completely utilized (most common food proteins, fats, carbohydrates, etc.) errors of fecal analysis are minimized by the fact that the error falls on the small unutilized residue. Thus if 10 per cent of the protein in a given diet remains unutilized an error of 10 per cent in the analysis would change the utilization value only 1 per cent. The iron analysis, which may be carried out much more accurately than this, does not, therefore, introduce a variation outside the natural limits of error of any procedure of this type.

The usefulness in gastrointestinal studies of the principle involved has previously been shown in connection with its use in obtaining an index of bacterial action in the intestine<sup>1</sup> and in studies of salivary digestion in the human stomach.<sup>2</sup> Its value in studies of intestinal absorption, digestion, and utilization in man and lower animals will be illustrated in the following communications.

#### SUMMARY.

A simplified method is presented for the determination of food digestibility and utilization. Iron oxide is added to the food

<sup>1</sup> Bergeim, O., *J. Biol. Chem.*, 1924-25, lxii, 45, 49.

<sup>2</sup> Bergeim, O., *Arch. Int. Med.*, 1926, xxxvii, 110.

and by determining the ratio of the amount of any given food substance to the amount of iron in the food and in the feces the percentage utilization may be calculated. Accurate account of food ingested, separation, and complete collection of feces are not essential for this method which thus becomes available in many cases where the more elaborate procedure would not be employed. The method is applicable to studies on small animals such as albino rats.





## INTESTINAL CHEMISTRY.

### V. CARBOHYDRATES AND CALCIUM AND PHOSPHORUS ABSORPTION.

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(Received for publication, June 28, 1926.)

It is held by certain observers that rickets is primarily a disturbance of calcium and phosphorus absorption inasmuch as the percentage of these elements excreted by way of the feces is high in rickets and this percentage is lowered by administration of antirachitic vitamin treatment with ultra-violet light, or suitable changes in the proportions of calcium and phosphorus in the diet, increased deposition of these elements in the bones being simultaneously observed. Even though rickets be not always due entirely to failure of calcium and phosphorus absorption as a result of a depression of an intestinal function or unsuitable character of the intestinal content, nevertheless when we have to deal with such a relatively insoluble salt as calcium phosphate and with diets none too abundantly supplied with any form of calcium it is necessary to give careful consideration to all of the factors which may influence calcium absorption. This is particularly true because of the fact that the antirachitic factor is not abundant in most of the common foods. Even on a diet of milk which is rich in calcium and phosphorus rickets not infrequently develops and we may expect some defect in calcium assimilation to exist in many cases where rickets is not demonstrated. Rickets being possible on a milk diet apparently in part because milk does not always contain any considerable excess of antirachitic vitamin, other alterations in the composition of milk such as are carried out constantly in infant feeding and which may influence calcium absorption must be considered. That administration of

antirachitic vitamin may overcome a rachitic tendency is not conclusive proof that this alone is always the best means of securing the desired result. At any rate in the modification of milks and generally in the preparation of diets it is desirable to know what influence any particular constituents of the diet may have on calcium absorption. The present investigation is devoted primarily to the study of the influence of common carbohydrates on calcium absorption. Other dietary constituents will be considered in more detail later.

In the present investigation the iron index procedure as suggested by the author for the study of gastrointestinal chemistry and function<sup>1</sup> was employed. This consists in the addition to the diet of a known amount of some substance such as ferric oxide. In studying calcium utilization the calcium : iron ratio is determined in the food and also in the feces or intestinal content. Iron is not appreciably absorbed; therefore the percentage calcium absorbed is readily calculated. For example if the Ca:Fe ratio of the food is 10:1 and of the feces 4:1 then the feces contain  $4 \div 10$  which is 0.4 or 40 per cent of the original calcium, and 100-40 or 60 per cent of the original calcium has been absorbed.

The present series of experiments was carried out on albino rats. Young animals weighing about 60 gm. were placed for a period of 25 to 30 days on the following diet: whole wheat 33, gelatin 15, wheat gluten 15, whole yellow corn 33, NaCl 1.0, CaCO<sub>3</sub> 3.0, agar-agar 0.5, and ferric oxide 0.2. The inorganic salts were first ground together in a mortar and then mixed thoroughly with the other ingredients all of which passed through sieves of at least 20 mesh. All cages used were scrupulously clean as traces of antirachitic food substances vitiate the results. Direct sunlight was excluded. During the last 10 days of the period each animal was kept in a separate cage. After this time the diets were varied by additions of various amounts of carbohydrates.

Calcium and phosphorus absorption were determined on the control diet for each rat during two successive 4 day periods and

<sup>1</sup> Bergeim, O., *J. Biol. Chem.*, 1924-25, lxxi, 45, 49; 1926, lxx, 29; *Arch. Int. Med.*, 1926, xxxvii, 110.

for the modified diets on successive 4 day periods. At the end of each 4 day period feces were collected, any showing contamination with urine being discarded. The rats were then placed on the diet of the next period, the feces of the following 2 days being discarded before collection began on the next 4 day period. Observations were made of the amounts of food ingested as failure to eat affects the results, but for many purposes the exact amounts of food eaten or feces resulting therefrom need not be considered.

Calcium, phosphorus, and iron were determined in the feces, and in the food only so often as the ratio of the elements named to each other was altered.

About half a gm. of the feces (not weighed) was ashed, with the help of a few drops of nitric acid to get rid of the last carbon, at not too high a temperature in a silica crucible (35 cc.). 10 cc. of HCl 1:1 was added and the mixture digested on a hot plate for 15 to 30 minutes to dissolve all iron and calcium. Little phosphorus was lost in ashing because of the excess of calcium carbonate present but controls using moist ashing procedures should be carried out where phosphorus absorption is to be studied. The ash solution was made up to approximately 25 cc. with distilled water.

For the calcium determination 2 cc. of the ash solution were measured into a 100 cc. Erlenmeyer flask, 25 cc. of water added, a drop of alizarin indicator, and ammonia to neutrality, then 3 cc. each of approximately 0.5 N HCl and of 2½ per cent oxalic acid. Then 10 cc. of 3 per cent ammonium oxalate were added and slowly with shaking 3 cc. of 20 per cent sodium acetate. The whole was transferred to centrifuge tubes, let stand for several hours, the supernatant fluid drawn off, the tube nearly filled with water, inverted against the palm of the hand, centrifuged, and the fluid again drawn off. A siphon previously described is convenient for this purpose.<sup>2</sup> 5 cc. of 5 per cent sulfuric acid were added, the mixture warmed in a bath to 65°, and titrated with about N/50 permanganate.

The number of mg. of calcium per cc. was calculated and divided by the number of mg. of iron found per cc. This figure was divided by the Ca:Fe ratio of the food and the result times

\* Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.

100 gives percentage of original calcium present. Subtracting from 100 gave the percentage of calcium absorbed.

For the determination of iron 1 cc. portions of ash solution were measured into large test-tubes, 8 cc. of water and 2 cc. of 2 per cent KCNS solution added. The solutions were compared with standards in a colorimeter. For standards 1, 2, 3, 5, and 8 cc. portions of standard ferric sulfate<sup>3</sup> solution containing 0.1 mg. of iron per cc. were measured into test-tubes, to each 1 cc. of 1:4 HCl was added and water to make 9 cc., then 2 cc. of KCNS solution. Mg. of iron per cc. were calculated.

For the determination of phosphorus to 1 cc. portions of solution in large test-tubes add 10 cc. of water and 2 drops of concentrated sulfuric acid. Prepare standards in test-tubes (2, 4, 7, 10 cc. of a solution containing 0.1 mg. of P per cc.) adding sulfuric acid and water to make volumes of 11 cc. Add 2 cc. of molybdate solution (5.0 per cent ammonium molybdate in N sulfuric acid) and 2 cc. of hydroquinone (20 gm. of hydroquinone and 1 cc. of concentrated sulfuric acid in 1 liter) to each and compare after a few minutes in a colorimeter. The phosphate standard contains 0.4394 gm. of dry monopotassium phosphate per liter. Calculate mg. of P per cc. of solution. Calculate percentage absorption of phosphorus as described under calcium.

### *Results.*

Results of experiments on carbohydrates are illustrated in the accompanying charts (Figs. 1 to 4).

*Lactose.*—The lactose used was a Schuchardt product further purified by two recrystallizations with alcohol and by a 24 hour extraction first with alcohol and then with ether. It was of course essential that the sugar should be free from antirachitic substance in appreciable amounts. That the results were not due to such contamination is believed to be assured by the method of purification, by the fact that the same results were obtained with the original lactose, and by the product further purified,

<sup>3</sup> Dissolve 0.7 gm. of ferrous ammonium sulfate (dried to constant weight) in 100 cc. of distilled water. Add 5 cc. of concentrated sulfuric acid. Warm slightly and add  $\text{KMnO}_4$  solution until oxidation is complete. Dilute to 1 liter. 1 cc. = 0.1 mg. of Fe.

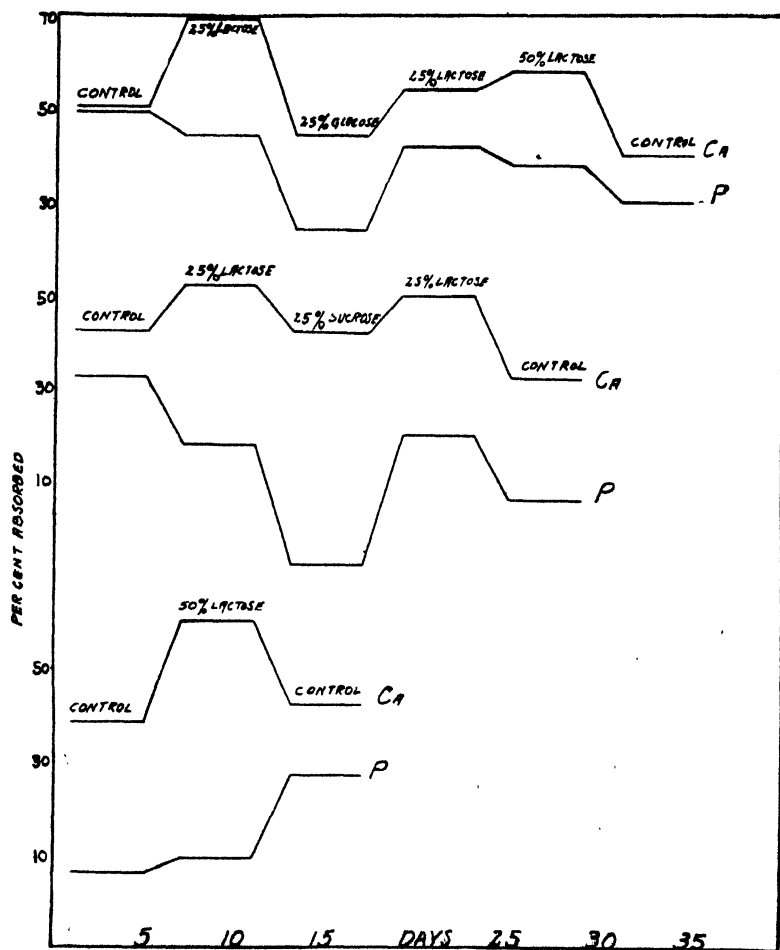


FIG. 1. Influence of lactose on calcium and phosphorus absorption.

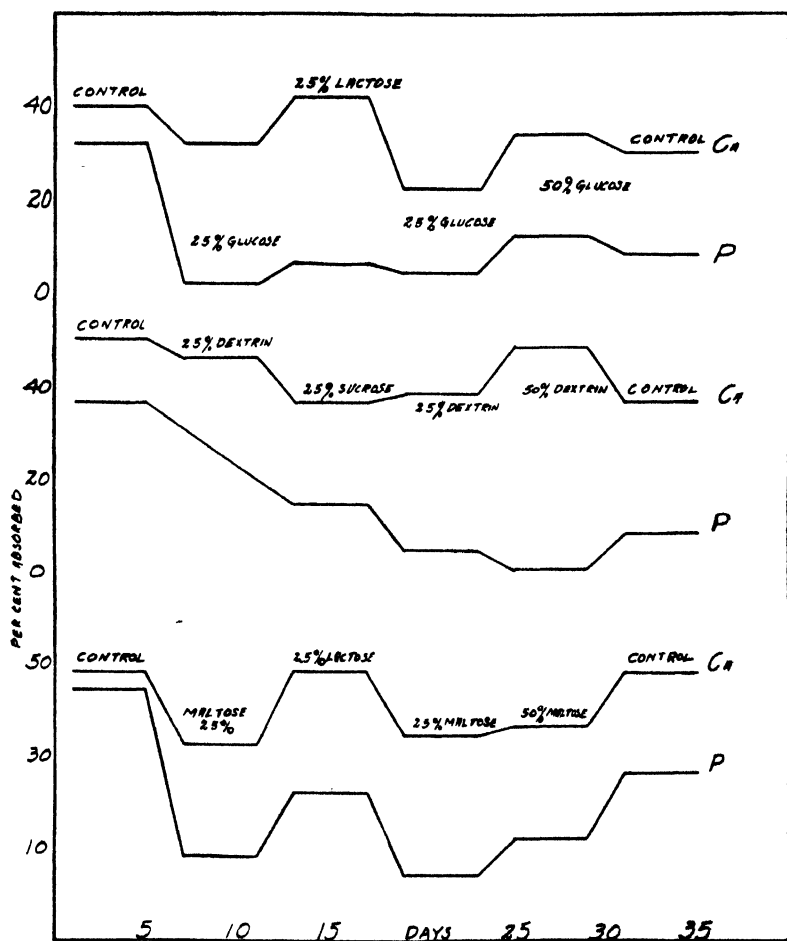


FIG. 2. Influence of sugars on calcium and phosphorus absorption.

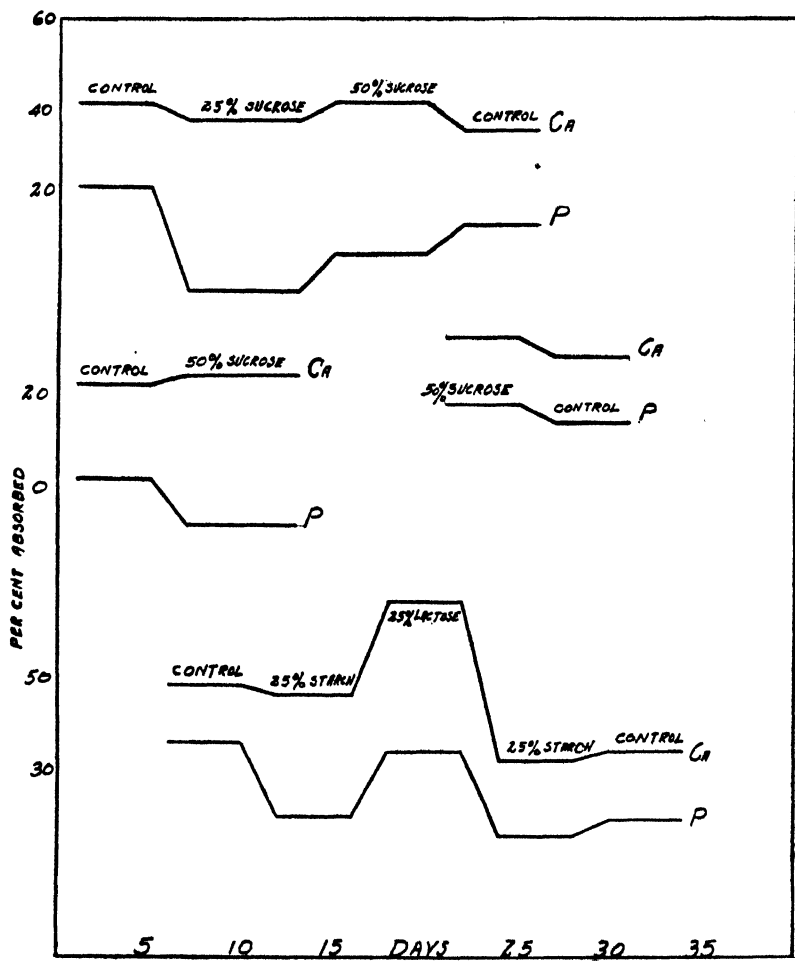


FIG. 3. Influence of sucrose and starch on calcium and phosphorus absorption.



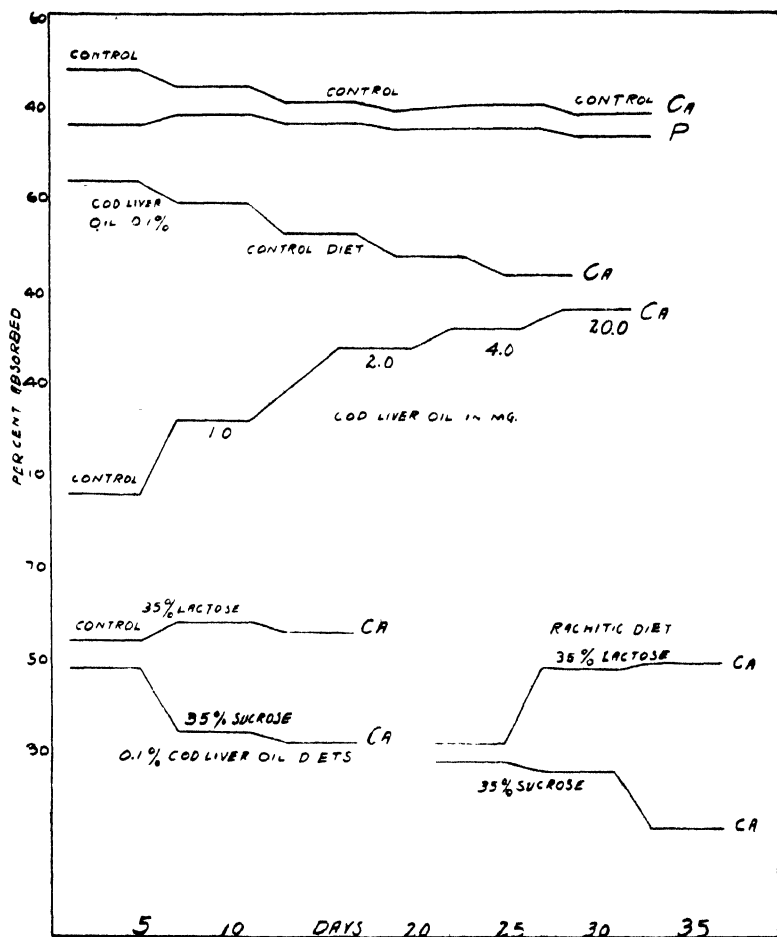


FIG. 4. Influence of cod liver oil, etc., on calcium and phosphorus absorption.

by the poverty of even pure milk fat in antirachitic substance, by the fact that dextrin gives somewhat similar results, by the observation that vitamin effects are carried over to subsequent periods while the effect of lactose is rapidly lost, and by the fact that lactose administration did not prevent the development of rickets on these diets.

From the charts it will be seen that lactose had a much more pronounced effect on calcium and phosphorus absorption than glucose, sucrose, maltose, or starch, bringing about definitely increased absorption. This was true on rachitic diets and also on diets containing cod liver oil. Phosphorus absorption was less affected than calcium absorption and in certain cases little or no effect was observed in spite of definitely increased calcium absorption.

*Dextrin.*—Dextrin added to the extent of 25 per cent of the diet produced little effect, but in amounts of 50 per cent a distinct increase in calcium absorption was noted. Dextrin was thus less effective than lactose but produced better results than the other carbohydrates tested. Little effect on phosphorus absorption was noted in either case.

*Sucrose, Glucose, Maltose, and Starch.*—In no case did 25 per cent of sucrose increase calcium absorption. 50 per cent had a slight effect. The same was true of phosphorus absorption except that the results were somewhat less favorable.

Glucose showed up unfavorably as compared with lactose and showed no improvement over the control. In the larger quantities glucose produced a more favorable effect.

Maltose compared unfavorably with lactose even when given in the larger amounts. Decreased rather than increased absorption was noted for both calcium and phosphorus.

Results with starch indicate little effect as compared with control diet. Lactose led to much increased calcium absorption.

The curve of an animal kept on the control diet throughout is given for comparison. Each animal is further a control upon itself where the diets are varied as in these experiments.

The rapid and pronounced effect of even very small amounts of cod liver oil on calcium absorption is also illustrated for comparison. The slow decline of absorption following withdrawal of this oil from the diet is also shown.

## DISCUSSION.

It is well known that increase of acidity markedly increases the solubility of such salts as calcium phosphate. Lactose when administered in considerable amounts is known to produce a distinctly acid condition throughout the gastrointestinal tract. Dextrin has a similar but lesser effect. Other common carbohydrates have little effect apparently because they or their products of hydrolysis are very rapidly absorbed. It has therefore seemed reasonable to suppose that lactose administration might by increasing the acidity of the intestinal contents also increase the absorption of calcium. Proof of this has however been mostly indirect. Thus Inouye<sup>4</sup> found that lactose in contrast with sucrose, glucose, and dextrin protected animals from experimental tetany presumably by keeping up the calcium content of the blood.

The present experiments show definitely that calcium absorption is markedly increased by lactose administration as contrasted with administration of glucose, sucrose, maltose, starch, and even dextrin. There does not appear to be any other reasonable explanation of these facts than that of the increased acidity of the intestinal contents which does exist and seems quite adequate to account for all of the observations.

The pronounced effectiveness of lactose in increasing calcium absorption should be considered in connection with the preparation of milks for infant feeding and generally in the planning of diets inasmuch as calcium is probably one of the limiting factors in the diets of many people. This does not necessarily mean that lactose is always the sugar of preference. Sugars more readily digested and absorbed and producing less fermentation in the bowel may be preferred for example in certain gastrointestinal disturbances. Nor will lactose take the place of antirachitic substance or of phosphorus in the diet. Where, however, calcium absorption is one of the main factors to consider the effectiveness of lactose in this direction should be borne in mind. In this connection the much higher proportion of lactose to calcium and phosphorus in human as compared with cow's milk is of interest. The fact that lactose is particularly effective also in reducing intestinal putrefaction is a further recommendation for its use. It is an interesting fact that the sugars which would most readily

<sup>4</sup> Inouye, T., *Am. J. Physiol.*, 1924, lxx, 524.

undergo alcoholic fermentation with a loss of food value to the organism are rapidly absorbed while lactose which undergoes fermentation with formation of lactic acid (an acid which forms glucose in the body) is only digested and absorbed slowly.

The apparent depression of absorption of calcium and phosphorus on certain of the high carbohydrate diets has not been explained. It is known however that strong sugar solutions depress gastric secretion of hydrochloric acid which might assist in calcium solution.<sup>5</sup> There exists an impression that high carbohydrate diets are unfavorable to calcium assimilation in some other way. There is of course on high carbohydrate diets a danger of inorganic and vitamin deficiency and sugars have a depressing effect on hunger and appetite.

The author desires to thank Mr. W. F. Tolar and Mr. E. O. Jodar for assistance in carrying out these experiments.

#### CONCLUSIONS.

1. A procedure for the study of calcium and phosphorus utilization is described.
2. Common carbohydrates were added to the diets of rachitic animals to make up 25 to 50 per cent of such diets, and calcium and phosphorus absorption studied.
3. Starch, glucose, fructose, and maltose in amounts of 25 per cent did not increase calcium or phosphorus absorption. When 50 per cent was added there was some increase in absorption.
4. Dextrin showed little effect in the smaller amounts but a distinct effect in the larger quantities.
5. Lactose even in the proportion of 25 per cent led to pronounced increases in the amounts of calcium and phosphorus absorbed, being in this respect far superior to glucose, sucrose, maltose, or starch and superior to dextrin. The effect was greater on calcium than on phosphorus absorption.
6. The influence of lactose and to a lesser extent of dextrin and other carbohydrates on calcium absorption is believed to be due to increased lactic acid fermentation in the intestines with resulting increased acidity of the intestinal contents. Lactose did not prevent the development of rickets on diets high in calcium but low in phosphorus and antirachitic substance.

<sup>5</sup> Miller, R. J., Bergeim, O., Rehfuess, M. E., and Hawk, P. B., *Am. J. Physiol.*, 1920, liii, 65.



## INTESTINAL CHEMISTRY.

### VI. A METHOD FOR THE STUDY OF ABSORPTION IN DIFFERENT PARTS OF THE GASTROINTESTINAL TRACT.

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By means of fecal analyses it is possible to estimate the completeness of digestion and absorption of ingested substances. This method does not tell us to what degree such digestion or absorption has taken place in each part of the gastrointestinal tract. Nor in this way can we learn much as to the conditions under which these processes normally take place or how they can be influenced. Certain substances such as calcium, phosphorus, and iron are excreted into as well as absorbed from the intestines. Here a complete picture can be obtained not by fecal analysis but only by quantitative examination of the intestinal contents at different levels. For this purpose the method of intestinal fistulas has been available. If a known amount of food be ingested and the residue from it be allowed to pass out through an intestinal fistula the degree of digestion or absorption that has taken place above the fistula can be estimated. By repeating the experiment using fistulas in different parts of the intestines a more complete picture can be obtained. The possibilities of this method of study have by no means been exhausted. That the use of polyfistular animals in this manner entails certain practical difficulties is apparent from the relative infrequency of their use in spite of the many unsolved problems of intestinal chemistry. A simpler method should therefore have its uses.

The principle of the present method is identical with that of the procedure previously described for the study of food utilization.<sup>1</sup> Iron oxide or some similar non-absorbable substance is

<sup>1</sup> Bergeim, O., *J. Biol. Chem.*, 1926, lxx, 29.

mixed with the food. The iron remaining constant, its estimation in the intestinal contents at different levels serves as a guide in determining the rate of absorption of other substances. Thus if the ratio of calcium to iron in the original food is 10:1 and in the contents of the middle portion of the small intestine is 7:1, then 70 per cent of the calcium must remain unabsorbed up to that point and 30 per cent must have been absorbed. If the ratio in the large intestine of the same animal be 9:1 it would appear that 20 per cent of calcium had been excreted into the gut.

The iron compounds should be chosen and diets prepared so that the iron will be most certain to follow the substances studied through the stomach and intestines. If the absorption of a readily soluble substance is being followed soluble iron salts such as the citrates may be used. In many cases a finely powdered pure ferric oxide or hydroxide will be found most convenient. Such a powder has been mixed for example with the calcium carbonate of a diet when calcium absorption was being studied. In following the digestion of the starch and protein of bread, powdered ferric oxide has been baked into the loaf. The digestion of raw starch has been followed by precipitating ferric hydroxide on a small portion of the starch. Starch granules so treated do not separate out from suspensions containing untreated starch. Other applications of this principle may be made. Obviously the food must be finely divided and thoroughly mixed. Where fecal analyses are being made as in utilization experiments such great precautions are not necessary as mixing occurs in the large intestine. Probably the chief danger of stratification occurs in the stomach, yet this difficulty was not met with in studies of salivary digestion in the human stomach.<sup>2</sup> The danger of stratification is not great in many cases. However all possible precautions should be taken. Checks and controls will show if the desired result is being attained. It is not necessary however that the intestinal content should be uniform in consistency. Varying dilutions with secretions or other fluids need not alter the ratio of iron to calcium or other food substances and we are concerned with this ratio and not with absolute amounts.

Key substances (iodides, urea, fat, etc.) have been used in

<sup>2</sup> Bergeim, O., *Arch. Int. Med.*, 1926, xxxvii, 110.

attempts to study gastric absorption and evacuation in human subjects but not with complete success.<sup>3</sup> Wildt<sup>4</sup> many years ago fed sheep wheat straw and used the silica naturally contained therein as an index of the absorption of other substances.

It is desirable in many cases to control carefully the method of feeding. If we wish to know what proportion of phosphorus is absorbed from a given diet in different parts of the tract the animal must be killed at a time when absorption is proceeding in a normal average manner throughout the intestines. This is best accomplished by feeding the animal the diet in question until all residues of former diet have been swept from the intestines (usually 24 hours or more) and then feeding the same diet at frequent intervals (every 4 hours or so), giving each time such an amount of food that the succeeding meal will also be eaten, and killing the animal a short time (2 to 3 hours) after the last food has been ingested. Food in various stages of digestion will then be found throughout the intestines. Of course if desired several animals may be used and each killed at a different time after food ingestion. If animals with a series of fistulas are used an iron-containing diet makes it possible to determine the percentage digestion, secretion, or absorption at different levels by the removal of small samples for analysis instead of the removal in each case of the total residue from a given meal. The same principle may of course be applied in connection with intestinal loops.

The application of the method is not restricted to studies of digestion or absorption. Any alterations in the chemical nature of the intestinal contents from whatever cause can be followed in this way. The analytical technique is the same as that previously described for utilization experiments.<sup>5</sup> Specific applications of the method will be presented in succeeding papers.

#### CONCLUSIONS.

A procedure is outlined for the study of absorption from the gastrointestinal tract. The ratio of the amount of ferric oxide

<sup>3</sup> Cascao de Anciaes, J. H., *Arch. Verdauungskr.*, 1924, xxxii, 343.  
Bloomfield, A. L., and Keefer, C. S., *Arch. Int. Med.*, 1926, xxxvii, 819.

<sup>4</sup> Wildt, E., *J. Landwirtschaft.*, 1874, xxii, 1; 1879, xxvii, 177.

<sup>5</sup> Bergeim, O., *J. Biol. Chem.*, 1926, lxx, 35.



or other unabsorbable substance to the amount of any other substance present is determined for the food and for the intestinal contents at different levels of the tract. The change in ratio is a quantitative index of the extent of absorption from or excretion into the gut. Digestive and other chemical changes may also be followed. The method may be applied to material obtained from the intestines of animals killed at the height of digestion or from intestinal fistulas and can be used with small animals such as the albino rat.

## INTESTINAL CHEMISTRY.

### VII. THE ABSORPTION OF CALCIUM AND PHOSPHORUS IN THE SMALL AND LARGE INTESTINES.

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(Received for publication, June 28, 1926.)

The problems involved in calcium and phosphorus absorption possess an unusual interest because of the fact that the di- and tricalcium phosphates are very insoluble in neutral solutions and because a condition of experimental rickets can be produced either by low calcium, high phosphorus or low phosphorus, high calcium diets, this condition involving a loss of calcium or phosphorus or both to the body by way of the intestinal tract. Especially noteworthy is the fact that minute amounts of anti-rachitic substance such as may be found in a few mg. of cod liver oil may swing the balance of these elements from strongly negative to strongly positive. At the present time controversy continues as to whether the defect in rickets lies in the inability of the bone cells to utilize calcium and phosphorus brought to them or in a failure of absorption of these elements from the intestines.<sup>1</sup> The study of the intestinal phase is complicated by the fact that calcium and phosphorus are not only absorbed from but are also excreted into the intestines so that fecal analyses represent only a summation of these processes. The procedure described by the author in which unabsorbable iron oxide is mixed with the diet and determined along with calcium and phosphorus in different parts of the intestinal tract<sup>2</sup> makes it possible readily to study absorption of these elements in different parts of the tract even in small animals such as the albino rat. The present paper

<sup>1</sup> Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, xx, 379. Robison, R., *Biochem. J.*, 1926, xx, 388.

<sup>2</sup> Bergeim, O., *J. Biol. Chem.*, 1926, lxx, 47.

presents some results on the influence of antirachitic substance on the absorption of calcium and phosphorus from different parts of the intestinal tract.

Six albino rats each weighing about 120 gm. were placed on a calcium-high phosphorus-low diet (wheat gluten 15, gelatin 15, whole yellow corn 33, whole wheat (soft) 33, NaCl 1, CaCO<sub>3</sub> 3, Fe<sub>2</sub>O<sub>3</sub> 0.25, all ground to pass a 20 mesh or finer sieve and thoroughly mixed, the inorganic salts being first ground together in a mortar. Especial care in grinding and mixing was necessary only for the food given during the last few days of the experiment.) The animals were kept on this diet for 25 days, a rachitic condition being induced. They were then divided into two groups of three

TABLE I.  
*Ca:P Atomic Ratios in Intestinal Contents.*

Part of intestines.	Rachitic animals.			Animals given cod liver oil.		
	R1	R2	R3	C1	C2	C3
Middle small. ....	0.34	0.61	0.44	0.30	0.30	0.96
Lower " ....	1.50	1.44	0.70	2.03	0.60	1.46
Cecum. ....	2.50	2.43	1.97	2.22	2.01	1.66
Upper large. ....	2.30	2.00	2.32	2.21	2.42	1.37
Lower " ....	3.30	2.55	1.73	1.45	2.25	2.02

each. The rats of one group were given 10 drops each per day of cod liver oil for 5 days and the other group 10 drops each of olive oil beside the usual diet. During the last 24 hours of the experiment the animals were fed individually pellets of the moistened diet at 10 p.m., 8 a.m., 12 noon, and 1 p.m. Sufficient food was not given at any time so that the animals would not consume the next meal. Each animal received in all 10 gm. of food. The animals were killed with chloroform at 5 p.m. The gastrointestinal tracts were removed and separated into stomach, middle small intestine (second and third quarters), lower small intestine (fourth quarter), cecum, upper large intestine, and lower large intestine. The contents of each section were transferred to silica crucibles and ignited at a low temperature. The ash was dissolved in HCl and calcium, phosphorus, and iron determined by methods previously described.<sup>3</sup>

<sup>3</sup> Bergeim, O., *J. Biol. Chem.*, 1926, lxx, 35.

Three other non-rachitic animals were fed in the same way a diet consisting of powdered milk 50, corn-starch 50, and ferric oxide 1. This diet is of course high in both calcium and phosphorus.

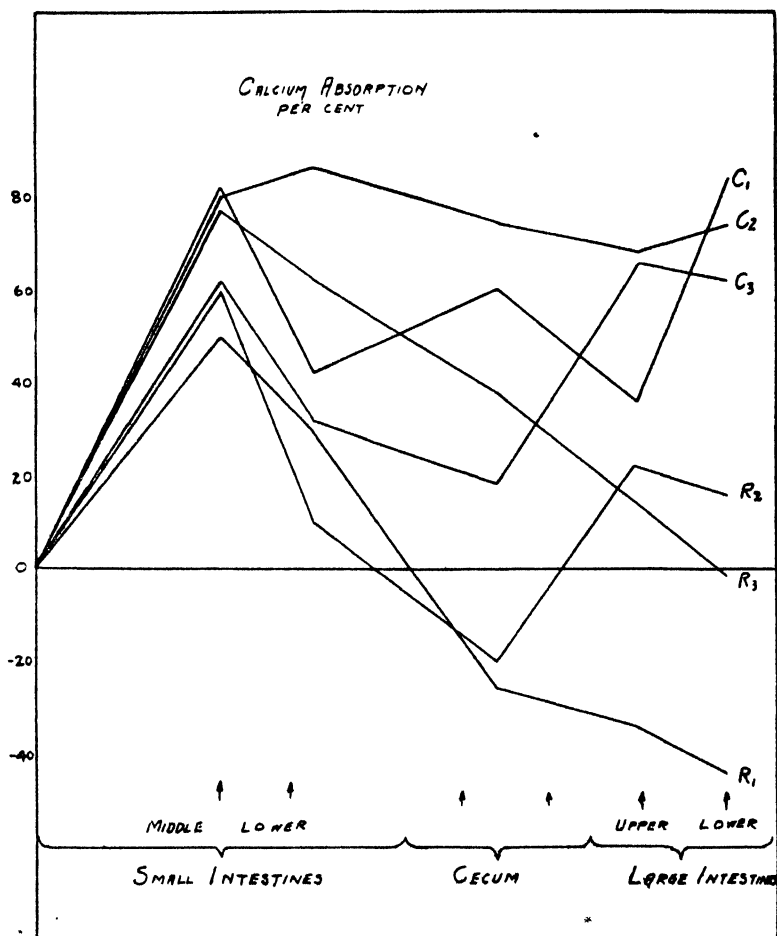


FIG. 1. Calcium absorption from intestines of rachitic animals (R) and animals given cod liver oil (C).

From the analyses the Ca:Fe and P:Fe ratios were calculated for each section of the intestines and also for the food and the percentage absorption of each element determined. Thus a

ratio of 8:1 in the food and 4:1 in the intestines indicated 50 per cent absorption. The results obtained are presented in Figs. 1 to 3. Results are expressed as percentages of the amounts of

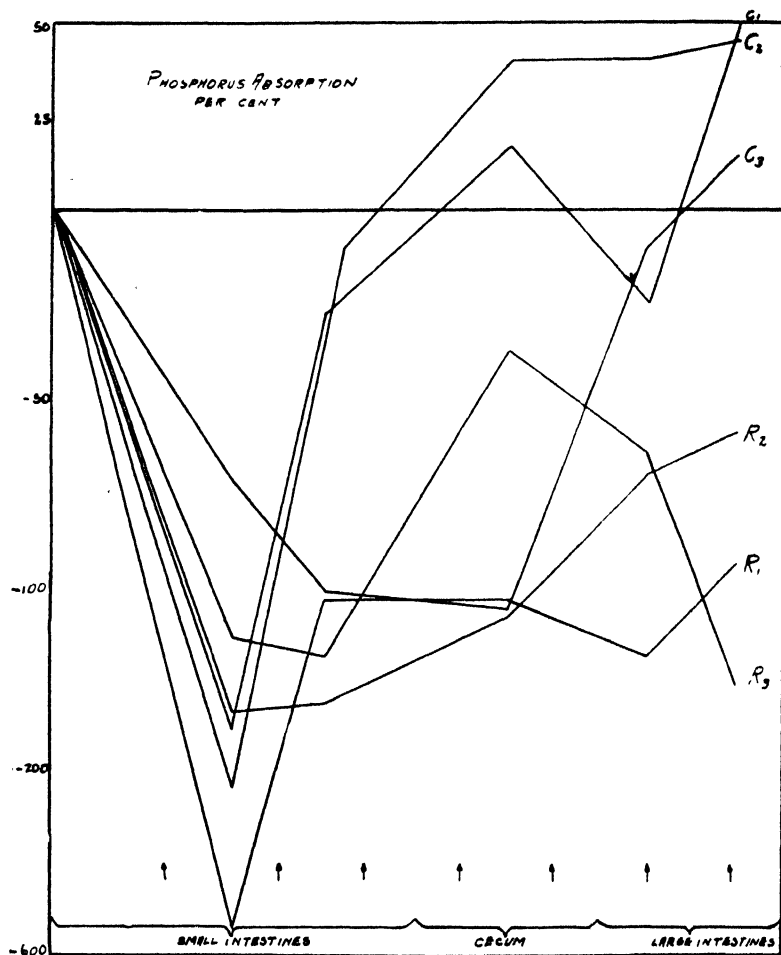


FIG. 2. Phosphorus absorption from intestines of rachitic animals (R) and animals given cod liver oil (C).

calcium and phosphorus in the food. As the Ca:P ratio in the food was 5.7:1 the phosphorus percentages would have to be divided by 5.7 to make them more absolutely comparable with those of calcium. The atomic ratios in the diet were Ca:P::4.4:1.

The atomic Ca:P ratios for the intestinal contents are given in Table I.

On the milk diet an acid condition generally prevailed throughout the intestines due presumably to lactic acid fermentation, and both calcium and phosphorus were absorbed to a considerable

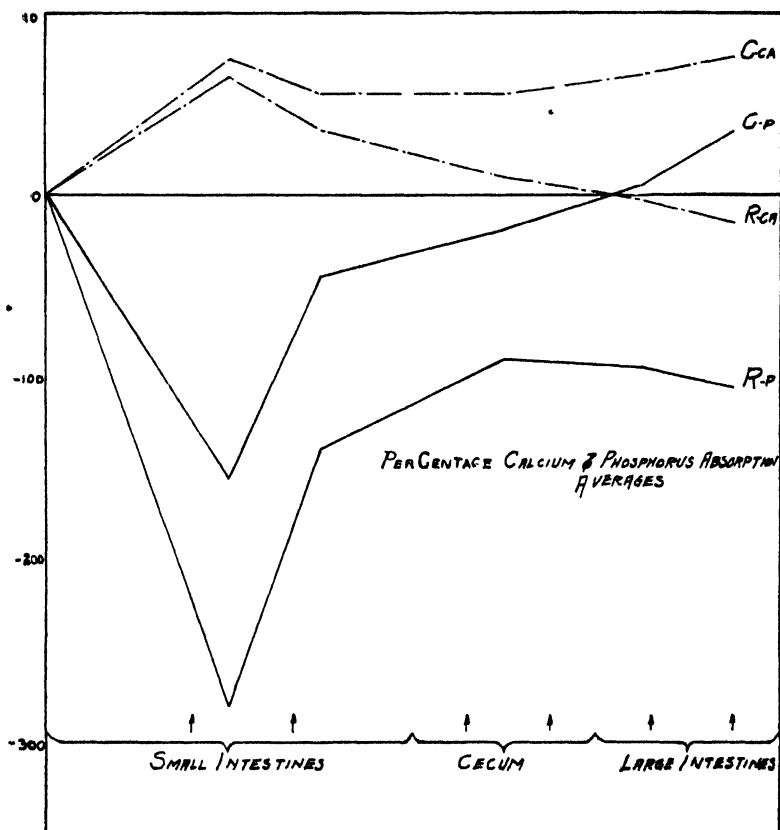


FIG. 3. Average absorption of calcium and phosphorus from intestines of rachitic animals (R) and animals given cod liver oil (C).

extent in the small intestines. Calcium and phosphorus were excreted into the cecum and large intestines, the final balance being positive or negative depending apparently upon the ability of the tissues of the animals to retain these elements.

From the data it appears:

1. Both normal animals (those fed cod liver oil) and rachitic

animals show a considerable degree of calcium absorption in the small intestines. On this diet therefore (a calcium-high phosphorus-low diet) the rachitic condition could not be due to a failure of calcium absorption.

2. Both normal and rachitic animals show a considerable excretion of phosphate into the small intestines. As calcium absorption took place most rapidly where this excretion of phosphate was most marked and hence the Ca:P ratio was lowest it would appear that the excretion of phosphate into the intestine was an important factor in promoting calcium absorption.

3. In the normal animals there was an approximate balance between excretion and absorption of calcium in the lower bowel so that a high positive calcium balance was maintained. Phosphorus was also reabsorbed so as to bring about a positive balance of this element.

In the rachitic animals on the other hand the excretion of calcium into the lower bowel was much more marked than absorption and led to very low retentions or losses of this element. Coincident with this marked excretion of calcium into the lower bowel there was a failure of phosphate to be adequately reabsorbed and hence considerable losses to the body resulted.

The results obtained support the following interpretation.

1. The secretion of phosphate into the gastrointestinal tract favors the absorption of calcium. This was suggested by the author some years ago on indirect evidence.<sup>4</sup>

2. In rickets calcium may be absorbed from the upper intestine but reexcreted into the lower intestine so that a loss of calcium to the body results. This reexcretion of absorbed calcium must be due to a failure of the bones to take up this element for purposes of calcification. As rachitic bones are able to take up calcium and phosphorus *in vitro*<sup>1</sup> the failure of calcification must lie at least in part outside the bone cells and be due to the low phosphate content of the blood which is so uniformly noted in rickets. This low blood phosphate is the result of the inability of the tissues of the body to maintain the proper concentration through breakdown of organic phosphates which they contain. That the body tissues are low in phosphorus in rickets has been shown.<sup>5</sup> Antirachitic vitamin would therefore appear to act by

<sup>4</sup> Bergeim, O., *Proc. Soc. Exp. Biol. and Med.*, 1914, xii, 22.

<sup>5</sup> McCann, G. F., and Barnett, M., *J. Biol. Chem.*, 1922, liv, 203.

facilitating breakdown of organic phosphates of the tissues, thus elevating the blood phosphate and enabling absorbed calcium to be deposited in the bones. This calcium being deposited in the bones is not reexcreted in the lower bowel to hinder phosphate absorption which is therefore favored so that the phosphate balance also becomes positive and healing of rickets begins. In rickets of the phosphorus-low type therefore tissues other than the bones or intestinal mucosa would appear to be predominantly concerned, although we should expect these tissues also to be affected, and in other types of rickets they may be primarily involved.

This view would correlate the hypothesis presented by us<sup>4</sup> as to the importance of phosphoric esterase in calcium absorption, calcification, and decalcification with the evidence presented in this paper, with the direct demonstration of hexose phosphatase in bone cells by Robison *et al.*<sup>1</sup> and with the finding of Shipley, Kramer, and Howland<sup>1</sup> that in experimental rickets the defect is not primarily in the bones. It is of interest also that interpretation of the parathyroid effect as involving an increased breakdown of organic phosphates of the tissues which we presented some years ago<sup>4</sup> is supported by the finding of Greenwald of a phosphate loss in long continued parathyroid treatment. The favorable effect of fasting on calcification in rickets may in part be due to the fact that blood phosphate will be less constantly drained away by intestinal content high in calcium and some more phosphate will be liberated from the breakdown of body tissues. Considerable more work is necessary for a solution of the questions raised.

#### CONCLUSIONS.

1. The absorption of calcium and phosphorus from different parts of the gastrointestinal tract of albino rats was studied. Animals rendered rachitic by phosphorus-low diets as well as such animals given cod liver oil showed a considerable degree of calcium absorption from the small intestine. The rachitic condition could not therefore be due to a failure of calcium absorption.

2. Both groups of animals showed a considerable secretion of phosphate into the upper tract. This secretion appears to be



an important factor in promoting calcium absorption as the latter was most rapid where the P:Ca ratio was highest.

3. The animals given cod liver oil showed a positive calcium balance throughout the intestines. Phosphorus secreted into the upper tract was absorbed in the lower intestines to produce an ultimate positive balance of this element also.

4. In the rachitic animals the calcium absorbed in the upper intestine was excreted into the lower intestine, leading to a negative or subnormal balance. Coincident with this marked excretion of calcium into the lower bowel there was a failure of phosphate to be adequately reabsorbed and hence a loss of the latter to the body.

5. The failure of absorbed calcium to be used in calcification is believed to be due to the low phosphate concentration of the blood. Antirachitic substance may act by elevating blood phosphate by promoting the breakdown of organic tissue phosphates thus leading to increased deposition of calcium with lessened excretion into the gut and consequent better absorption of phosphate therefrom.

## THE PRODUCTION OF CONJUGATED GLYCURONIC ACIDS IN DEPANCREATIZED DOGS.

BY ARMAND J. QUICK.

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(Received for publication, June 24, 1926.)

Little is known concerning the possible relationship of glycuronic acid to carbohydrate metabolism although it is chemically an oxidation product of glucose. The most commonly accepted view is that glycuronic acid is produced only in response to the presence of certain types of aliphatic and aromatic compounds which contain either a hydroxyl group, or a radical which can in the body be either reduced or oxidized to an alcohol or a phenol.<sup>1</sup> Its synthesis is, therefore, looked upon as the product of a detoxication mechanism rather than an intermediary compound in carbohydrate metabolism. In fact some investigators do not even believe that glycuronic acid is derived from a carbohydrate precursor.

Many of the earlier experiments devised to determine the possible source of glycuronic acid depended upon ascertaining whether a fasting animal still had the power to synthesize the compound (1, 2). In view of our present knowledge concerning the tenacity with which the body conserves its store of glycogen and its power to convert protein to glucose and glycogen, these experiments prove nothing one way or the other. Mayer (3) claimed that in fasting rabbits the production of glycuronic acid in response to ingested camphor was greatly diminished but that the normal output of the conjugated acid could be restored by the administration of glucose. Von Fenyvessy (4) was not able to duplicate these results. On the contrary, he found that there was no decrease in glycuronic acid synthesis in response to cam-

<sup>1</sup> A number of aromatic acids, notably benzoic acid, are also conjugated with glycuronic acid.

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phor, phenol, or chloral, even after a rabbit had been fasted 12 days, and he, furthermore, failed to find any increase in conjugated glycuronic acid following the feeding of glucose. Mandel and Jackson (5) reported that glucose actually diminished glycuronic acid excretion in fasting dogs following the administration of camphor, whereas a meat diet caused an increased production.

TABLE I.

Dog 7, weight 10 kilos. Operated March 18. No food or insulin after March 21.

Date.	Excretion in 24 hrs.			D:N	D + G:N*	Remarks.
	Sugar.	Nitrogen.	Glycuronic acid (expressed as glucose).			
1928	gm.	gm.	gm.			
Mar. 24	8.30	3.90	3.40†	2.13	3.00	Approximately 4.5 gm. of sodium benzoate fed.
" 25	9.55	3.32		2.88		
" 26	4.21	3.52	3.11‡	1.20	2.08	4 gm. of sodium benzoate fed.

\* Dextrose + glycuronic acid (expressed as glucose): nitrogen.

† Total benzoic acid, 3.12 gm.; free benzoic acid, 0.10 gm.; benzoic acid as hippuric acid, 0.71 gm.; benzoic acid as glycuronic acid monobenzoate calculated, 2.31 gm. (equivalent to 3.63 gm. of glycuronic acid or 3.40 gm. of glucose).

‡ Total benzoic acid, 2.74 gm., free benzoic acid, 0.12 gm.; benzoic acid as hippuric acid, 0.51 gm.; benzoic acid as glycuronic acid monobenzoate calculated, 2.11 gm. (equivalent to 3.36 gm. of glycuronic acid or 3.11 gm. of glucose).

Loewi (6) studied the problem of the origin of glycuronic acid by means of phlorhizinized dogs. He worked on the premise that if glucose and glycuronic acid have the same precursor, then the administration of a glycuronogenic drug like camphor should in the diabetic organism cause a decrease in sugar excretion corresponding to the glycuronic acid produced. He found that there was either no decrease in sugar excretion or a greater drop in the nitrogen so that his D:N ratio was increased rather than decreased. He concluded from these results that glucose and

glycuronic acid had different precursors, and that, therefore, glycuronic acid was not derived from glucose. Mayer (3) severely criticized these results and pointed out that Loewi neglected to take into consideration the possibility of glycogen which might still be stored in the body. Since these animals were fasted and given phlorhizin for 2 weeks, it hardly seems possible that the glycogen still remaining could have affected Loewi's results sufficiently to be of any significance. Mayer's other criticism, namely, that accurate conclusions cannot be deduced from polariscopic determinations on urine containing unchanged levo-phlorhizin, levo conjugated glycuronic acid, and dextro-glucose, seems more

TABLE II.

Dog 8, weight 11 kilos. Operated March 30. No insulin or food after April 2.

Date.	Excretion in 24 hrs.			D:N	D + G:N	Remarks.
	Sugar.	Nitrogen.	Glycuronic acid (expressed as glucose).			
1926	gm.	gm.	gm.			
April 4	21.2	5.65		3.75		
" 5	20.0	6.16		3.24		
" 6	11.5	4.64	2.76	2.48	3.07	5 gm. of borneol fed.

The animal contracted distemper a few days after the operation and was ill during the experimental period. Death ensued in about 30 hours after the administration of borneol. On autopsy there was grossly no pancreatic tissue and no indication of pregnancy.

pertinent. Loewi, as well as the other earlier investigators, was handicapped, as was pointed out in a previous paper (7), by the lack of satisfactory analytical methods for conjugated glycuronic acids.

Lewin (8) also working on phlorhizinized animals reported that there was an increased output of phenol and indoxyl accompanied by a corresponding rise in the excretion of conjugated glycuronic acids. Mayer (9) could not corroborate these results; in fact, he stated that he could find no glycuronic acid in the urine of phlorhizinized rabbits.

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An interesting contribution to the subject of glycuronic acid production in phlorhizin diabetes was made by Schüller (10) who actually succeeded in isolating phlorhizin glycuronic acid in rabbits' urine after administering excessively large doses of phlorhizin. The conjugated compound was distinctly less effective in producing diabetes than the free drug, which is evidence for the view that glycuronic acid serves the function of a detoxifying agent. Strangely enough the glucoside portion of phlorhizin was not

TABLE III.

Dog 9, weight 17.3 kilos. Operated April 10. No food or insulin after April 13.

Date.	Excretion in 24 hrs.			D:N	D + G:N	Remarks.
	Sugar.	Nitro- gen.	Glycu- ronic acid (expressed as glucose).			
1926	gm.	gm.	gm.			
Apr. 14	62.7	10.20		6.20		
" 15	36.9	11.20		3.30		
" 16	41.4	13.55		3.06		
" 17	39.1	12.6	6.35*	3.10	3.61	Approximately 6 gm. of sodium benzoate fed.
" 18	16.5†	3.88	Trace.	4.25		

\* Total benzoic acid 5.65 gm.; free benzoic acid, 0.36 gm.; benzoic acid as hippuric acid, 0.99 gm.; benzoic acid as glycuronic acid monobenzoate calculated 4.30 gm. (equivalent to 6.84 gm. of glycuronic acid or 6.35 gm. of glucose).

† The determination was made on about a 12 hour specimen of urine collected before the dog died. It was contaminated with vomitus, since the dog vomited every time it drank water.

oxidized to the corresponding glycuronic acid, but instead a molecule of glycuronic acid was conjugated through one of the free phenolic groups of the phloretin. This is not in accord with the theory of Sundwik (11) and Fischer and Piloty (12) that the glucoside is formed as an intermediary compound which undergoes further oxidation to glycuronic acid. Other investigators especially Hildebrandt (13) and Hämäläinen (14) have found that ingested glucosides are excreted as the corresponding

glycuronic acids. In interpreting these results one must bear in mind the possibility that the glucosides may undergo hydrolysis in the body and that the phenolic portion may be subsequently conjugated with glycuronic acid.

The conflicting results obtained both on fasting and on phlorhizinized animals have failed to throw much light either on the

TABLE IV.

Dog 10, weight 8.2 kilos. Operated April 10. No insulin or food after April 13.

Date.	Excretion in 24 hrs.			D:N	D + G:N	Remarks.
	Sugar.	Nitrogen.	Glycuronic acid (expressed as glucose).			
• 1928	gm.	gm.	gm.			
Apr. 15	24.00	3.60		6.65		
" 16	6.65	2.69		2.47		
" 17	4.55	2.68		1.70		
" 18	0.94*	3.67	4.35†	0.26	1.44	5 gm. of sodium benzoate fed.
" 19	11.80	5.88		2.01		
" 20‡	14.85	6.66		2.22		200 gm. of meat fed.
Apr. 25	8.60	2.96		2.90		
" 26	7.55	2.91		2.60		
" 27	4.65	2.56	1.52	1.82	2.41	3 gm. of borneol fed.
" 28	6.16	2.58		2.39		
" 29	7.08	3.02		2.35		

On autopsy there was grossly no pancreatic tissue nor any indication of pregnancy.

\* Urine volume, 530 cc.  $\alpha$  observed in a 1 dm. tube,  $+0.08^\circ$ .

† Total benzoic acid, 3.70 gm.; free benzoic acid, 0.23 gm.; benzoic as hippuric acid 0.62 gm.; benzoic as glycuronic acid monobenzoate calculated, 2.95 gm. (equivalent to 4.7 gm. of glycuronic acid or 4.35 gm. of glucose).

‡ The animal was again given insulin and food until April 25.

mechanism of glycuronic acid formation or on its relationship to protein and carbohydrate metabolism. No studies have been made on the synthesis of glycuronic acid in pancreatic diabetes. Such studies, especially with satisfactory quantitative methods for conjugated glycuronic acid which are now available, seemed promising and were undertaken. Dogs were chosen for this

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work since it is not possible to remove completely the pancreas from a rabbit. The statement is often made that the synthesis of conjugated glycuronic acid is better developed in the herbivora than in the carnivora, but recent studies on the formation of glycuronic acid monobenzoate and unpublished work on borneol glycuronic acid definitely show that the dog can produce glycuronic acid as readily and abundantly as the rabbit. The two glycuronogenic drugs chosen for the work were benzoic acid and

TABLE V.

Dog 11, weight 7.8 kilos. Operated May 6. No food or insulin after May 9.

Date.	Excretion in 24 hrs.			D:N	D + G: N	Remarks.
	Sugar.	Nitrogen.	Glycuronic acid (expressed as glucose).			
1926	gm.	gm.	gm.			
May 11	12.6	3.12		4.04		
" 13	11.2	4.16		2.69		
" 14	8.05	4.07	1.77	1.98	2.41	3 gm. of borneol fed.
" 15*	11.4	4.54	Trace.	2.51		
May 30	3.04	2.16		1.41		
" 31	2.65	2.38	1.59	1.11	1.78	3 gm. of borneol fed.
June 1	3.25	2.07		1.57		

\* Dog was again given insulin and food until May 22. On May 27 it was given an injection of a small dose of sodium lactate, as part of another experiment.

borneol, for either one is excreted largely combined with glycuronic acid, and can be given in moderately large doses without producing any observable toxic or pronounced physiological effects.

### EXPERIMENTAL.

Female dogs were employed. After the removal of the pancreas<sup>2</sup> the dogs were given insulin twice daily and kept on a diet

<sup>2</sup> All the operations were performed by Dr. Joshua E. Sweet, Laboratory of Surgical Research, University of Pennsylvania.

consisting of lean beef and sucrose. The addition of a small amount of powdered pancreatin to the food seemed to be definitely beneficial in aiding digestion. The insulin was usually withdrawn on the 4th day, and the animal fasted. On the 3rd or 4th day of fasting when the D:N ratio was about 3 or less, borneol or sodium benzoate was given by stomach tube. By incorporating these drugs in a 0.5 per cent solution of agar, it was possible to prevent vomiting which was one of the most serious difficulties encountered especially in feeding sodium benzoate.

#### *Analytical Methods.*

*Glucose.*—The Shaffer-Hartmann method was employed. A polariscopic examination of the urine was usually made to check the values obtained. When the urine contained glycuronic acid monobenzoate, which also reduces directly, glucose was calculated from the difference between the total reducing power and the reducing power of glycuronic acid monobenzoate (expressed in terms of glucose).

*Borneol Glycuronic Acid.*—The method used was the same as that described for menthol glycuronic acid (7) with the exception that the Shaffer-Hartmann instead of the Benedict method was employed.

*Glycuronic Acid Monobenzoate.*—The amount of glycuronic acid monobenzoate was calculated from the difference between the total benzoic acid and the benzoic acid combined as hippuric acid plus free benzoic acid. Total benzoic acid was determined by the method of Kingsbury and Swanson (15), free benzoic acid by the method of Raiziss and Dubin (16), and hippuric acid by the procedure recently described by the author (17).

#### DISCUSSION.

The question whether a pancreatic diabetic dog is still able to produce glycuronic acid can be emphatically answered in the affirmative. Although the dogs studied were presumably completely diabetic and had been fasted for several days they still possessed the power to produce conjugated glycuronic acid with apparently the same ease as that of a normal well fed dog. Since the glycogen stores in these animals must have reached a very



low level, one can conclude that the glycuronic acid, like glucose, was derived from the proteins of the body. If the same portion of the protein molecule yields both glycuronic acid and glucose, any production of glycuronic acid should be accompanied by a corresponding decrease in urinary sugar. In other words, there should be a distinct fall in the D:N ratio, but the D + G:N (dextrose + glycuronic acid:nitrogen) ratio should be roughly the same as the D:N ratios before and after the experiment. An examination of the protocols shows that this is the case. If the data on Dog 10 (Table IV) be studied, one finds that the ingestion of 5 gm. of benzoic acid causes a diminution of the urinary sugar from 4.55 gm. to 0.94 per 24 hours even though the nitrogen output was increased. This caused a drop in the D:N ratio of 1.7 of the previous day to 0.26. If the 4.55 gm. of glycuronic acid which is equivalent to 4.2 gm. of glucose be taken into consideration and the D + G:N ratio calculated, a value of 1.44 is obtained. This figure, although somewhat low, is in the range of the D:N ratios obtained before and after this particular experiment. A further experiment on the same dog feeding borneol in place of benzoic acid gave similar results. Again there was a marked decrease in the excretion of sugar with a corresponding fall of the D:N ratio. The value of the D + G:N ratio, however, approximated fairly closely the D:N ratios obtained on the days when no drug was administered. In all of the experiments except one the production of glycuronic acid was accompanied by a decrease in urinary glucose. This leads one to conclude that glycuronic acid and the glucose which is produced in a fasting diabetic animal have the same precursor, and that whenever there is a demand on the organism for glycuronic acid it is produced at the expense of the potential glucose. It is significant that the production of glycuronic acid is not always accompanied by an increase in urinary nitrogen. This can be interpreted to mean that the organism does not have to catabolize additional protein, but can synthesize moderately large amounts of glycuronic acid from the protein which it is required to break down for its maintenance under the conditions of the experiment.

The results obtained in this study are in distinct disagreement with the findings of Loewi (6). One can hardly attribute this

difference to the fact that he employed phlorhizinized dogs whereas depancreatized dogs were used in the present work. It seems much more plausible that the cause can be ascribed to the use of camphor by Loewi for inducing the synthesis of glycuronic acid. Camphor has a pronounced physiological action and when used in such large doses as Loewi employed, it is very likely to produce disturbances in metabolism which can complicate the study of the production of glycuronic acid. It must also be remembered that camphor is a ketone and must, therefore, be reduced to the alcohol before it can be conjugated with glycuronic acid. Any irregularity in the oxidative or reductive processes of the organism is, therefore, apt to influence indirectly the synthesis of glycuronic acid. It might be mentioned here that Dog 9, which was the only dog in the present series which failed to show a drop in urinary sugar on the day it received benzoic acid, died (apparently as the result of pernicious vomiting) within 36 hours after the administration of the drug.

In this work no constant D:N ratio could be obtained. The ratio dropped continuously and in a number of cases became less than 2. Weber, Briggs, and Doisy (18) as well as other investigators have obtained similar series of values on depancreatized dogs. The cause for this continuous decrease is not known, but there may be the possibility that part of the protein which can go to sugar and is excreted may be converted to substances needed in the economy of the organism. Thus, in this present work it was demonstrated that glycuronic acid can be formed from the glycogenic part of the protein molecule. Recently Weber, Briggs, and Doisy (18) found that depancreatized dogs still retained the power of forming lactic acid. Similar observations have also been made on phlorhizinized dogs by Loebel, Barr, Tolstoi, and Himwich (19). These authors also noted that in certain of their experiments the D:N ratios dropped to a definitely lower level following the administration of adrenalin. They suggest as a possible explanation for this that a part of the carbohydrate fraction of the protein molecule may be retained to form a new glycogen store.

Evidence is thus accumulating which indicates that the diabetic organisms can still utilize, at least in part, the carbohydrate fraction of the protein molecule. From the results which are

being obtained on intermediary carbohydrate metabolism as well as from the opinions expressed at various times by investigators in this field, a hypothesis can be formulated which is as follows: All the sugar-forming amino acids may perhaps yield one common simple chemical substance such as lactic acid. This substance can be converted by the organism either to glycogen (lactic acid precursor) or to glucose which in the diabetic animal is not utilized and is excreted in the urine. According to this theory glucose is not formed as an intermediary compound in the conversion of lactic acid to glycogen. This appears to be in accord with recent findings that the diabetic animal can still produce lactic acid as well as lactic acid precursor, although the organism has no power to utilize glucose. In the normal animal, it is possible, as has been suggested by Loebel *et al.*, that glucose may be degraded along two distinct paths, an oxidative and an anoxidative. In the oxidative process the end-products are carbon dioxide and water, while in the anaerobic process the end-product is lactic acid which can be converted to glycogen. Of course, it is well known that the reaction, lactic acid to glucose, can readily take place and lactic acid perhaps is changed to glucose before it undergoes oxidation. In the diabetic organism glucose can no longer be utilized; therefore, the glycogen stores must be maintained by the protein. The mechanism  $\text{glycogen} \rightleftharpoons \text{lactic acid}$  does not appear to be disturbed, and the protein must replace only the lactic acid that is lost in the cycle. If the assumption is correct that all the sugar-forming amino acids form one common intermediary substance in their conversion to glucose, it is reasonable to suppose that glycuronic acid may be synthesized from this or some other common component, which in the diabetic animal is derived from protein and in the normal animal from the degradation of glucose.

The writer takes this opportunity to express his thanks to Dr. J. E. Sweet whose cooperation made possible this research.

#### SUMMARY.

The depancreatized dog can still produce glycuronic acid in amounts similar to those produced by normal dogs.

The production of glycuronic acid is accompanied by a corresponding decrease in the urinary sugar indicating that glycuronic acid and glucose have the same precursor; and that, when there is a demand on the organism for glucuronic acid, it is produced at the expense of the potential glucose. Since the glucose produced in total diabetes during fasting is generally believed to be solely derived from protein, it can be concluded that the diabetic organism can still utilize that portion of the protein molecule which ordinarily goes to glucose for the synthesis of glycuronic acid.

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## STUDIES IN CARBOHYDRATE METABOLISM.

### IX. CONTINUED INVESTIGATIONS INTO THE INFLUENCE OF INSULIN AND MUSCLE TISSUE ON GLUCOSE IN VITRO.

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(Received for publication, April 26, 1926.)

#### INTRODUCTION.

In an earlier contribution (1924) we have shown that insulin and fresh finely minced muscle tissue added simultaneously to a solution of  $\alpha$ - $\beta$ -glucose at 37°C. produces a diminution in the specific rotation angle of the glucose in the solution. In a later article (1925) we showed that the observed change must be due to an unknown form of glucose which we have called new-glucose. But new-glucose is not formed by the addition of insulin alone nor muscle tissue alone to a solution of  $\alpha$ - $\beta$ -glucose. Although both components are equally important for the formation of new-glucose, it is not certain that the *simultaneous* action of insulin and muscle tissue on  $\alpha$ - $\beta$ -glucose is an essential condition for the production of new-glucose. Other possibilities can be imagined; namely, either (a) that the active body in muscle tissue by itself can transform  $\alpha$ - $\beta$ -glucose into a form which can then be changed into new-glucose by insulin without further intervention of the muscle tissue or (b) that insulin can transform  $\alpha$ - $\beta$ -glucose into a form which can then be changed into new-glucose by the active substance in muscle tissue without further intervention of insulin. Or it may be that the muscle tissue and insulin can interact in such a way that the one factor alone after the removal of the other can transform  $\alpha$ - $\beta$ -glucose into new-glucose. If such is the case we must assume either (c) that the insulin occurs in the form of a proferment and is changed by the muscle tissue into an active ferment; or (d) that the muscle tissue yields a proferment which is turned into an active form by the insulin.

As all information about the first stage in the transformation of glucose in the organism is of importance for understanding the carbohydrate metabolism in the organism, we have investigated the above possibilities in the present work.

TABLE I.

*200 Cc. of 1 Per Cent  $\alpha$ - $\beta$ -Glucose and 15 Gm. of Fresh Finely Minced Muscle Tissue Shaken for 2 Hours at 37°C. to Which Were Then Added 20 Units of Insulin.*

Experiment No	Time after addition of insulin.	Ventzke degrees directly observed.	Glucose.		Specific rotatory value.
			Rotation value.	Reduction value.	
	<i>hrs.</i>		<i>per cent</i>	<i>per cent</i>	
1	$\frac{1}{2}$	1.29	0.422	0.419	+52.9°
	1	1.21	0.399	0.405	+52.1°
	2	1.23	0.405	0.407	+52.3°
2	$\frac{1}{2}$	1.29	0.422	0.425	+52.0°
	1	1.33	0.433	0.437	+52.0°
	2	1.24	0.407	0.407	+52.5°
3	$\frac{1}{2}$	1.25	0.409	0.411	+52.3°
	2	1.19	0.394	0.392	+52.7°
4	$\frac{1}{2}$	1.23	0.405	0.409	+52.0°
	1	1.23	0.405	0.410	+52.1°
	2	1.25	0.409	0.412	+52.3°

### *Investigations and Results.*

#### *A. Can Muscle Tissue Alone Transform $\alpha$ - $\beta$ -Glucose into a Form Which Can Then Be Changed into New-Glucose by Insulin without Any Further Intervention of Muscle Tissue?*

The method used in this series of experiments was the following: To 200 cc. of isotonic sodium chloride solution containing 1 per cent  $\alpha$ - $\beta$ -glucose were added at 37°C. 15 gm. of fresh muscle tissue. The mixture was put in a shaking machine in a thermostat at 37°C. After 2 hours 20 units of insulin were introduced. From our earlier experiments we knew that the active substance in muscle tissue was rendered inactive after 2 hours' standing in physiological salt solution. If any new-glucose was detected

after the addition of insulin it could only be explained by the fact that the fresh muscle tissue had converted  $\alpha$ - $\beta$ -glucose into a form which insulin could act upon. From time to time after the insulin was added samples of the mixture were withdrawn. They were put in the previously described dialysis apparatus. After half an hour's dialysis the specific rotation angle of the glucose in the dialysate was determined both by rotation and reduction estimations. The experimental results are given in Table I. The determinations were done as previously described. The rotation value was the mean of twenty readings. The reduction value was the mean of four analyses by the potassium ferricyanide method. (As to technical details see Paper VI of this series.)

From Table I it appears that values were found in all the samples whose deviations from the specific rotation angle,  $+52.5^\circ$ , of  $\alpha$ - $\beta$ -glucose were within the experimental error. *It is thus shown that the active substance in muscle tissue does not convert  $\alpha$ - $\beta$ -glucose into a form which insulin can afterwards change into new-glucose.*

*B. Can Insulin Convert  $\alpha$ - $\beta$ -Glucose into a Form Which Can Then Be Changed into New-Glucose by the Active Substance in Muscle Tissue Without the Intervention of Insulin?*

In this series of experiments the method was as follows: To 200 cc. of a 1 per cent solution of  $\alpha$ - $\beta$ -glucose at  $37^\circ\text{C}$ ., 20 units of insulin were added. After 2 hours' action the solution was put in a dialysis apparatus at  $37^\circ\text{C}$ . In half an hour's time the dialysate was removed and 15 gm. of fresh finely minced muscle tissue were added to it. After 1, 2, and 3 hours' action samples were removed for the determination of the specific rotation angle of the glucose after renewed dialysis in the usual manner. The results are recorded in Table II. As will be seen from the table no change in the specific rotation angle of the glucose was observed in these experiments either. *The results show that insulin does not convert  $\alpha$ - $\beta$ -glucose into a form which can then be changed into new-glucose by the active substance in muscle tissue.*

The experiments also prove that demonstrable amounts of insulin have not passed through the collodion membrane during the half hour that the first dialysis lasted. If this had happened there would have been an opportunity for the formation of new-glucose



TABLE II.

*Dialysate from 200 Cc. of 1 Per Cent  $\alpha$ - $\beta$ -Glucose Plus 20 Units of Insulin to Which Were Added 15 Gm. of Fresh Finely Minced Muscle Tissue.*

Experiment No.	Time after addition of insulin.	Ventske degrees directly observed.	Glucose.		Specific rotatory value.
			Rotation value.	Reduction value.	
	<i>hrs.</i>		<i>per cent</i>	<i>per cent</i>	
1	1	1.28	0.420	0.420	+52.5°
	2	1.27	0.417	0.418	+52.4°
	3	1.21	0.398	0.399	+52.4°
2	1	1.12	0.369	0.373	+52.0°
	2	1.22	0.402	0.406	+52.1°
	3	1.20	0.396	0.400	+52.0°
3	1	1.37	0.446	0.451	+52.1°
	2	1.33	0.435	0.435	+52.5°
	3	1.34	0.437	0.434	+52.8°

TABLE III.

*150 Cc. of Physiological Salt Solution, 15 Gm. of Muscle Tissue, and 20 Units of Insulin to Which Were Added after 2 Hours 20 Cc. of 5 Per Cent  $\alpha$ - $\beta$ -Glucose Solution.*

Experiment No.	Time after addition of insulin.	Ventske degrees directly observed.	Glucose.		Specific rotatory value.
			Rotation value.	Reduction value.	
	<i>hrs.</i>		<i>per cent</i>	<i>per cent</i>	
1	1	1.27	0.417	0.412	+52.9°
	2	1.32	0.430	0.424	+53.3°
	4	1.36	0.444	0.440	+52.9°
2	$\frac{1}{2}$	1.15	0.383	0.386	+52.1°
	2	1.23	0.405	0.405	+52.5°
3	1	1.29	0.422	0.425	+52.0°
	2	1.26	0.413	0.409	+52.9°

when the muscle tissue was added. Only the *negative* results we obtained in our experiments, therefore, could decide the problem before us by this method.

*C and D. Can muscle Tissue and Insulin Act upon One Another in Such a Way That One of Them Alone, After the Removal of the Other, Can Convert  $\alpha$ - $\beta$ -Glucose into New-Glucose?*

The experiments in this last series were carried out as follows: To 150 cc. of a physiological salt solution at 37°C. 15 gm. of fresh finely minced muscle tissue and 20 units of insulin were added simultaneously. After 2 hours 50 cc. of a 5 per cent solution of  $\alpha$ - $\beta$ -glucose were introduced. As already mentioned we knew from our previous experiments that the substance in muscle tissue which in conjunction with insulin can transform  $\alpha$ - $\beta$ -glucose into new-glucose becomes *inactive* after being kept in physiological salt solution for 2 hours.

After the addition of the glucose solution samples of the mixture were removed from time to time for the determination of the specific rotation angle of the glucose. The experiments are recorded in Table III. As appears from the table no real deviation from the specific rotation angle of  $\alpha$ - $\beta$ -glucose +52.5° was detected.

The results showed that with the method used new-glucose was not formed. We can, therefore, say that no reciprocal action of the two factors, insulin and the active body in muscle tissue, upon one another can be detected 2 hours after the two components come in contact. With the experimental technique employed in these investigations it has thus *not* been possible to isolate the two actions (that of insulin and muscle tissue) with respect to time. *Within the interval* (2 hours) which the technique requires it cannot be shown that insulin influences the active substance in muscle tissue or that the latter acts on insulin in such a way that the activated substance can convert  $\alpha$ - $\beta$ -glucose into new-glucose by itself. By this process of exclusion we are justified in maintaining that the actions of insulin and muscle tissue on the transformation of  $\alpha$ - $\beta$ -glucose into new-glucose proceed simultaneously.

Finally it should be mentioned that control experiments were made in connection with the three series of investigations, in which insulin and fresh muscle tissue were both added to a solution of  $\alpha$ - $\beta$ -glucose at 37°C. In all the control tests a decrease in the specific rotation angle was demonstrated in agreement with the quantitative relations we found in our first work on the subject.

The control experiments proved that the insulin solution employed was active.

*Remarks.*

The main result of our experiments is, then, that the two factors, insulin and the active substance or principle in fresh finely minced muscle tissue, must act simultaneously (*i.e.* not in two measurably different tempi) on  $\alpha$ - $\beta$ -glucose when it converts this substance into new-glucose.

In a discussion of our earlier published investigations into carbohydrate metabolism Ahlgren has proposed to call the active body or principle in the muscles "glukomutin." Although we fully appreciate the practical advantages of giving this property of the muscles a definite name, we feel certain misgivings in accepting the term suggested by Ahlgren.

The expression glukomutin signifies literally that the muscle tissue possesses the property of changing (mutating) the glucose. If Ahlgren means that the muscle tissue itself transforms the glucose so that it can be attacked by insulin, this does not seem to be entirely supported by earlier investigations, and hardly tenable in view of the experiments recorded here. If, on the other hand, the author wishes merely in a general way to give expression to the fact that the muscle tissue only takes a share in converting glucose into new-glucose, we could with equal right give insulin the name glukomutin, so that this expression would really become a common term for the two substances which are able to transform glucose. We think, therefore, that it will be more reasonable for the time being to use a more neutral expression for the active substance or principle in muscle tissue, and we propose to use the expression "*insulin complement*." The name has the advantage that it covers what we at present know, while it cannot be said literally to indicate processes about which nothing is known.

RÉSUMÉ.

1. It has been shown that the active substance in muscle tissue does not transform  $\alpha$ - $\beta$ -glucose into a form which insulin can then alone convert into new-glucose.
2. It has been shown that insulin does not change  $\alpha$ - $\beta$ -glucose

into a form which can then be converted into new-glucose by the active substance in muscle tissue.

3. Within the period of the experiment it cannot be shown that insulin and the active substance in muscle tissue influence one another in such a way that one of them can convert  $\alpha$ - $\beta$ -glucose into new-glucose by itself. The action of the two factors on  $\alpha$ - $\beta$ -glucose must, therefore, be simultaneous within the period of 2 hours.

4. It is proposed for the present to call the active substance or principle in muscle tissue "insulin complement."

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## STUDIES IN CARBOHYDRATE METABOLISM.

### X. INVESTIGATIONS INTO THE OCCURRENCE OF INSULIN COMPLEMENT IN THE MUSCLES OF WARM BLOODED AND COLD BLOODED ANIMALS.

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#### INTRODUCTION.

In our first work on carbohydrate metabolism (1) we showed that a substance or principle (which we have proposed to call insulin complement) was present in the muscles of the guinea pig and mouse which, at 37°C., in conjunction with insulin, was capable of converting the ordinary form of glucose ( $\alpha$ - $\beta$ -glucose) into a new unknown form which we have proposed for the present to call new-glucose. The muscle tissue was inactive after keeping for 2 hours, and had also lost its power at a temperature of 20°C. In later papers (2) we proved that the transformation of the glucose which occurred *in vitro* was identical with the action of insulin in the normal human organism and in patients treated with insulin.

This introduced a method with the help of which it was possible to study *in vitro* the first step of the transformation of glucose. In addition to the direct significance of this observation in the study of carbohydrate metabolism, especially in patients with diabetes mellitus, it suggests, naturally, various problems of a more general biological nature. To these problems belongs the question whether insulin complement is specific for muscle tissue or whether it is also present in other tissues of the body, the question of its distribution in the animal and vegetable kingdom, and

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also the question whether it is bound up with the structural integrity of the cells, as well as its behavior towards various physical and chemical influences. We have investigated these different problems partly in association, as many questions encroach so much upon one another that in planning the experiments and clinical investigations the essentials had to be broad in their scope. In presenting the results, however, it is natural to confine oneself to the several questions, and in this paper we shall chiefly give an account of the problem of the presence of insulin complement in the muscle of warm blooded and cold blooded animals.

#### EXPERIMENTAL.

The animals whose muscles we have investigated were chosen so as to represent certain of the principal groups of the animal kingdom. The previous investigations on the warm blooded animals have been supplemented with experiments on the muscles of rats and rabbits. The toad family is represented by some experiments on the muscles of frogs. In the case of the fishes the muscles of the cod were examined, and the crustaceans are represented in the series by muscles from the lobster. All the animals were killed immediately before the experiments were made. The arrangement of the experiments was, on the whole, the same as in our first investigations, apart from differences with respect to the temperature at which the experiments were carried out.

#### *Technique.*

Immediately after the animal was killed a definite mass of muscle was taken out and instantly finely minced. Next 15 gm. of the muscle together with 20 units of insulin were added to 200 cc. of an isotonic solution containing 2 per cent  $\alpha$ - $\beta$ -glucose. The mixture was then shaken for 2 hours at 20°C. in the case of the cold blooded animals and at 37°C. in the case of the warm blooded ones. A portion of the mixture was then dialyzed for half an hour in accordance with the previously described modification of our first method. The reduction and rotation values of the glucose in the clear dialysate obtained in this way were estimated. The reduction value was determined as the mean of four analyses

by the Hagedorn and Norman Jensen method. The rotation value was the mean of twenty readings. For further technical details the reader is referred to Paper VI (3) of this series, where a full account of the technique is given.

### Results.

The results of our experiments are all given in Table I. In Columns 3 and 4 the glucose content of the dialysate is recorded, calculated from the rotation and reduction values respectively. All these values exhibit the same marked difference which we

TABLE I.

*Specific Rotation Value of Glucose in the Dialysate from a Mixture of Glucose, Insulin, and Muscle from Various Classes of Animals.*

* Source of muscle.	Ventzke degrees directly observed.	Glucose immediately after dialysis.		Specific rotation value.	Ventzke degrees directly observed.	Glucose after multirotation.		Specific rotation value.	Temperature of experiment.
		Rotation (calculated).	Reduction.			Rotation (calculated).	Reduction.		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		per cent	per cent			per cent	per cent		°C.
Rabbit...	2.23	0.732	0.982	+39.1°	3.00	0.985	0.985	+52.5°	37
Rat.....	1.83	0.601	0.743	+42.5°	2.28	0.748	0.746	+52.6°	37
Frog.....	2.03	0.666	1.155	+31.4°	3.27	1.073	1.110	+50.8°	20
" ....	2.45	0.804	1.180	+36.7°	3.50	1.149	1.173	+51.5°	20
Cod.....	2.96	0.972	1.288	+40.0°	3.90	1.281	1.287	+52.3°	20
Lobster..	1.12	0.367	0.470	+40.9°	1.44	0.472	0.470	+52.8°	20

demonstrated in our first paper, the reduction values being considerably greater than the corresponding rotation values.

In Column 5 the specific rotation angle, calculated from the rotation and reduction values measured immediately, is recorded. The values vary between +31.4 and +42.5°. The rotation value is followed in the usual manner until the multirotation is finished. The reduction value is then determined again. These results are entered in Columns 7 and 8 of Table I. It appears that the reduction value has remained unaltered while the rotation value has risen and has now reached the same magnitude as the reduc-



tion value. In accordance with this the specific rotation value at this time is found to be the same as the value of the  $\alpha$ - $\beta$ -glucose ( $+52.5^\circ$ ), which indicates that the new-glucose formed has gradually reverted entirely to the original form,  $\alpha$ - $\beta$ -glucose.

#### DISCUSSION.

The results thus show that in the muscle tissue of cold blooded animals belonging to different classes of the animal kingdom a substance or principle (insulin complement) is also found which possesses the property that, in conjunction with insulin, it can convert  $\alpha$ - $\beta$ -glucose into the form we call new-glucose. These experiments make it probable that at any rate the first step in carbohydrate metabolism takes place in the same manner throughout the animal kingdom. It may be specially pointed out that while the muscle tissue of warm blooded animals is inactive at  $20^\circ\text{C}$ ., this is not the case with that of the cold blooded animals.

#### RÉSUMÉ.

1. The substance or principle (insulin complement) demonstrated by us in the muscles of warm blooded animals, which in conjunction with insulin is capable of converting  $\alpha$ - $\beta$ -glucose into new-glucose, has also been detected in the muscles of cold blooded animals representing different classes of the animal kindgom.

2. Unlike the insulin complement from the muscles of warm blooded animals, that from cold blooded animals is active at  $20^\circ\text{C}$ .

3. The experiments show that the first step in carbohydrate metabolism is the same throughout the animal kingdom.

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## **STUDIES IN CARBOHYDRATE METABOLISM.**

### **XI. INVESTIGATIONS INTO THE OCCURRENCE OF NEW-GLUCOSE IN THE COURSE OF THE FERMENTATION OF $\alpha$ - $\beta$ -GLUCOSE.**

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#### **INTRODUCTION.**

\* *Metabolism, Glycolysis, and Fermentation.*

In earlier articles (1) we have shown that new-glucose is an essential link in the transformation of glucose which takes place in the animal organism. In all probability new-glucose is the first step in the transformation which glucose has to take whether it is burnt up into carbonic acid and water, converted into glycogen, or deposited as fat. Besides this transformation of glucose which occurs in the animal body, other ways in which glucose is broken up are met with in nature. Of special biological interest are the two processes called glycolysis and fermentation. Of these the first very probably occurs in the human organism but to such a small extent that it can play no rôle in the energy transformation of the organism. Since, moreover, it was proved in the paper referred to that the enzymatic breaking down of glucose in the glycolytic process also occurs in the blood of diabetics although to a rather less degree than in normal blood, new-glucose cannot be an essential step in the process.

Regarding the nature of the process taking place when glucose ferments, it is uncertain whether in its initial stage it passes through the same links as those occurring in the breaking down of glucose which takes place in the animal organism, or whether it pursues another course. In this connection it may be recalled that

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Collip (2) in 1923 demonstrated that in various respiring plants, as well as in yeast, a hormone resembling insulin can be detected which he calls glucokinin.

Besides the general biological importance of the problem of the nature of the fermentation, a point more particularly of medical interest is associated with it. Since Leo (3) (1898) recommended the administration of yeast in the treatment of diabetes, this therapy has been discussed time and again in medical literature. Practically every author who has subjected this method of treatment to scientific investigation and criticism has arrived at the conclusion that it is useless in all serious cases of diabetes mellitus. On the other hand it is admitted by different observers that the administration of different forms of yeast by the mouth may be followed at any rate by a temporary decrease in the glycosuria. The yeast treatment has never been extensively employed in diabetic therapy, but it is used by a number of patients themselves as a household remedy. No scientific basis for the yeast treatment exists.

From our earlier investigations it appears, as mentioned, that in its decomposition in the organism during metabolism, glucose must pass through the stage which we have called new-glucose. As, moreover, we have worked out a method for demonstrating new-glucose *in vitro* one is led to investigate by this means whether new-glucose can be detected during glucose fermentation in accordance with what occurs during the transformation of glucose in the organism with the help of insulin and insulin complement. In order to study the question further we have attempted to determine in a series of experiments whether new-glucose can be demonstrated during the fermentation of  $\alpha$ - $\beta$ -glucose.

#### EXPERIMENTAL.

##### *Technique.*

After a solution of  $\alpha$ - $\beta$ -glucose (the strength of the solution generally varied between 5 and 10 per cent) was started fermenting by one of the methods referred to below, a sample was withdrawn and put in a dialyzing membrane at a temperature of 20°C. When dialysis had proceeded for half an hour the dialysate was removed and its glucose content determined both by reduction

and rotation estimations. The reduction estimations were done by the Hagedorn and Norman Jensen method. The rotation values were the mean of twenty readings.

TABLE I.

Experiment No. (1)	Conditions of experiment. (2)	Time at 37°C. before dialysis. (3)	Ventake degrees directly observed. (4)	Glucose.		Specific rotation value. (7)
				Rotation value. (5)	Reduction value. (6)	
		<i>min.</i>		<i>per cent</i>	<i>per cent</i>	
1	10 gm. fresh bottom yeast + 50 cc. 5 per cent $\alpha$ - $\beta$ -glucose.	10	1.01	0.331	0.337	+51.3°
2	10 gm. fresh top yeast + 50 cc. 10 per cent $\alpha$ - $\beta$ -glucose.	40	0.72	0.236	0.240	+51.6°
3	4 gm. dried bottom yeast + 50 cc. boiled juice of fresh bottom yeast + 10 cc. 50 per cent $\alpha$ - $\beta$ -glucose.	30	1.97	0.644	0.660	+51.2°
4	10 gm. fresh bottom yeast plasmolyzed with 1.5 cc. ether + 35 cc. boiled juice of fresh bottom yeast + 10 cc. 5 per cent $\alpha$ - $\beta$ -glucose.	30	2.09	0.686	0.673	+53.4°
5	30 cc. maceration juice of dried bottom yeast + 10 cc. 20 per cent $\alpha$ - $\beta$ -glucose.	60	1.77	0.575	0.579	+52.2°
6	35 cc. maceration juice of dried bottom yeast, suspended in boiled juice of fresh top yeast + 8 cc. 50 per cent $\alpha$ - $\beta$ -glucose.	120	2.75	0.893	0.890	+52.6°
7	30 cc. expressed juice of fresh bottom yeast + 5 cc. 60 per cent $\alpha$ - $\beta$ -glucose.	30	3.94	1.294	1.282	+52.9°

### *Experimental Conditions.*

As appears from Column 2 of Table I, fermentation of  $\alpha$ - $\beta$ -glucose was produced by a variety of methods; namely, with the help of (1) fresh bottom yeast, (2) fresh top yeast, (3) dried bottom yeast to which was added the boiled juice of fresh bottom yeast,

(4) fresh bottom yeast plasmolyzed with ether and then mixed with the boiled juice of fresh bottom yeast, (5) juice obtained by maceration of dried bottom yeast, (6) maceration juice of dried bottom yeast suspended in the boiled juice of fresh top yeast, and (7) the expressed juice of fresh bottom yeast. The special experimental conditions will be seen from the table. In Column 3 is recorded the time the fermentation has lasted at 37°C. before the sample for dialysis at 20°C. was withdrawn.

The bottom yeast used in the experiments was kindly supplied to us from the Fermentation Laboratory at New Carlsberg, while the top yeast was the ordinary commercial baker's yeast. We ourselves prepared the dried bottom yeast by ordinary desiccation of the fresh bottom yeast in the air for 4 days. The juice obtained by maceration of the bottom yeast was prepared according to von Lebedew's (4) directions. Finally the expressed juice of fresh bottom yeast was prepared by Buchner's (5) method. The preparations were made through the kindness of Cand. pharm. Gad-Andresen at the laboratory of the Medicinal Compagni.

### Results.

The results are all recorded in Columns 4 to 7 of Table I. In Columns 5 and 6 the values of the glucose concentration in the dialysate, calculated on the basis of the rotation and reduction values, respectively, are entered. It was found in all the experiments without exception that the rotation and reduction values were identical. In accordance with this the specific rotation angle in each experiment showed values which coincided with that of  $\alpha$ - $\beta$ -glucose (+52.5°). It follows from this *that in no case was the presence of new-glucose or any other kind of sugar different from  $\alpha$ - $\beta$ -glucose demonstrated during the course of the fermentation process.*

### DISCUSSION.

The presence of new-glucose could not be detected during the fermentation of  $\alpha$ - $\beta$ -glucose produced by a number of different methods. These results are very much against new-glucose being a connecting link in the process of glucose fermentation, as we found it was in the decomposition of glucose during metabolism in the animal organism. The reason that the experiments cannot

give a decisive answer to this question, however, is because we are unable entirely to rule out the possibility that any new-glucose momentarily formed is further transformed. This possible transformation would then have to take place so rapidly—*in statu nascendi*—that the new-glucose could not succeed in passing out into the dialysate.

#### RÉSUMÉ.

1. New-glucose cannot be detected during the fermentation of glucose produced by a variety of different methods.

2. It is, therefore, very improbable—although not finally settled—that the fermentation of glucose proceeds with new-glucose as a connecting link in the process. The fermentation of glucose in its early stage is thus fundamentally different from the breaking down of glucose in the animal organism.

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## STUDIES IN CARBOHYDRATE METABOLISM.

### XII. INVESTIGATIONS INTO THE PROPERTIES OF INSULIN COMPLEMENT.

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#### INTRODUCTION.

In previous communications it was shown that in the muscles of warm blooded and cold blooded animals there is a principle or substance which in conjunction with insulin changes  $\alpha$ - $\beta$ -glucose into new-glucose. We have proposed the name of insulin complement for this principle or substance. So far we have only made a short study of its properties. We have already shown, however, that the insulin complement obtained from the muscles of warm blooded animals loses its activity when the fresh finely minced muscle is kept suspended in isotonic salt solution for 2 hours at 37°C. or is exposed to a temperature of 20°C. The insulin complement from the muscles of cold blooded animals is, on the other hand, active at 20°C. In the present paper we shall report the results of some experiments planned with the object of further investigating the properties of insulin complement, in the hope of getting a more thorough knowledge of its nature.

We have studied the following points: (a) is insulin complement identical with the coferment demonstrated by Meyerhof in muscle; (b) can it be extracted with water; (c) will it stand boiling; and (d) is it bound to the intact cell structure or does it occur in the tissue fluid?

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*A. Is Insulin Complement Identical with the Coferment  
Demonstrated by Meyerhof in Muscle?*

Meyerhof has shown (1918) that a coferment can be obtained from the muscles of cold blooded and warm blooded animals, which is identical with the coferment occurring in the expressed juice of yeast, whose presence in the yeast juice Harden and Young have shown is essential to the power of the latter to ferment sugar. In the articles referred to Meyerhof showed that yeast extract which was rendered incapable of producing fermentation by removal of the coferment, recovered its power to ferment sugar on the addition of muscle coferment. Meyerhof prepared muscle coferment by heating finely minced muscle with an equal

TABLE I.

*Action of Boiled Muscle Juice and Insulin on  $\alpha$ - $\beta$ -Glucose in Vitro.*

Experiment No.	Conditions of experiment.	Ventzke degrees directly observed.	Glucose.		Specific rotation value.
			Rotation value.	Reduction value.	
			<i>per cent</i>	<i>per cent</i>	
1	11 cc. boiled juice of frog muscle + 12 cc. 2.5 per cent $\alpha$ - $\beta$ -glucose + 10 units insulin...	1.55	0.508	0.495	+53.8°
2	18 cc. boiled juice of frog muscle + 18 cc. 3 per cent $\alpha$ - $\beta$ -glucose + 20 units insulin...	1.80	0.590	0.596	+52.0°
3	10 cc. boiled juice of frog muscle + 10 cc. 3 per cent $\alpha$ - $\beta$ -glucose + 10 units insulin...	1.96	0.644	0.646	+52.5°

weight of distilled water to the boiling point. The filtrate of this extract, the so called boiled muscle juice, contains the muscle coferment.

In order to decide whether the insulin complement which we demonstrated in the muscle of warm blooded and cold blooded animals was identical with Meyerhof's muscle coferment, the following experiment was performed. The boiled muscle juice of the frog was prepared according to Meyerhof's directions. This muscle juice was then mixed with a solution of  $\alpha$ - $\beta$ -glucose and insulin added. The details of the experiment appear in Table I.

After 2 hours action at 20°C. a sample was removed and put in a dialysis apparatus. After half an hour's dialysis the glucose content of the dialysate was estimated, both by reduction determinations (Hagedorn and Norman Jensen's method) and by rotation determinations (mean of twenty readings).

It will be seen from the results in Table I that no change has been produced in the glucose as the specific rotation angle is found to be that of  $\alpha$ - $\beta$ -glucose, +52.5°. It follows from these experiments that muscle coferment, prepared according to Meyerhof's instructions, cannot in conjunction with insulin convert  $\alpha$ - $\beta$ -glucose into new-glucose or in any other way alter the constitution of glucose. We can conclude, therefore, *that the insulin complement present in muscle is not identical with Meyerhof's muscle coferment.*

#### *B. Can Insulin Complement Be Extracted with Water?*

One of the characteristics which Meyerhof demonstrated for muscle coferment was that it could be extracted with water. As insulin complement and muscle coferment both occur in muscle tissue without, as just shown, being identical, one is led to investigate whether the property of being extractable by water is common to the two substances. To settle this question the following experiment was carried out. 15 gm. of freshly minced frog muscle were extracted for 10 minutes with running water. The muscle was then put in a mixture of 50 cc. of 5 per cent  $\alpha$ - $\beta$ -glucose and 50 cc. of 0.45 per cent sodium chloride solution. To this were added 20 units of insulin. After shaking for 2 hours at 20°C. a sample was removed which was dialyzed for 30 minutes at the same temperature. The glucose concentration in the dialysate was then determined in the usual manner by the rotation value. The results are given in Table II.

The results immediately after the dialysis was finished show the usual change in the specific rotation value which is observed when  $\alpha$ - $\beta$ -glucose is converted into new-glucose. A further proof that new-glucose was formed is that multirotation occurred. After the multirotation had come to an end the difference between the rotation and reduction values vanished and the two values corresponded with the first determined reduction value. It appears

from the experiments that insulin complement cannot be removed from finely minced muscle by careful washing, contrary to what happens in the case of Meyerhof's muscle coferment.

TABLE II.  
*Action of Extracted Frog Muscle and Insulin on  $\alpha$ - $\beta$ -Glucose in Vitro.*

Conditions of experiment.	Ventzke degrees directly observed.	Glucose immediately.		Specific rotation value immediately.	Ventzke degrees directly observed.	Glucose after multirotation.		Specific rotation value after multi-rotation.
		Rotation.	Reduction.			Rotation value.	Reduction value.	
15 gm. extracted frog muscle + 50 cc. 5 per cent $\alpha$ - $\beta$ -glucose + 50 cc. 0.45 per cent NaCl solution + 20 units insulin.	1.57	per cent 0.517	per cent 0.730	35.5°	2.22	per cent 0.730	per cent 0.735	+52.2°

TABLE III.  
*Action of Muscle Tissue Heated to 70° (for 2 Minutes) and Insulin on  $\alpha$ - $\beta$ -Glucose in Vitro.*

Conditions of experiment.	Ventzke degrees directly observed.	Glucose.		Specific rotation value.
		Rotation value.	Reduction value.	
15 gm. minced guinea pig muscle + 20 cc. salt solution heated for 2 min. to 70°C., after which 20 units insulin, 100 cc. $\alpha$ - $\beta$ -glucose, and 100 cc. 0.9 per cent NaCl solution were added.	1.97	per cent 0.647	per cent 0.650	+52.3°

### C. Can Insulin Complement Resist Heating?

As stated Meyerhof prepared muscle coferment by boiling finely minced muscle, a fact which shows that this substance is to a certain extent thermostable. In order to determine how insulin complement behaves in this respect the following experiment was

made. 15 gm. of fresh finely minced guinea pig muscle were heated with 20 cc. of physiological salt solution to 70°C. and kept there for 2 minutes. After cooling to 37°C. 20 units of insulin and 100 cc. of 2 per cent  $\alpha$ - $\beta$ -glucose and 80 cc. of 0.9 per cent sodium chloride solution were added. After 2 hours shaking at 37°C. a sample was withdrawn for dialysis, and after half an hour's dialysis the glucose concentration in the dialysate was estimated by the rotation and reduction values. It appears from the results, which are given in Table III, that corresponding values were found for the amount of glucose determined by the two methods and the specific rotation value agreed with that of  $\alpha$ - $\beta$ -glucose.

TABLE IV.

*Action of Expressed Muscle Juice (Prepared according to Buchner's Directions) and Insulin on  $\alpha$ - $\beta$ -Glucose in Vitro.*

Conditions of experiment.	Ventake degree directly observed.	Glucose.		Specific rotation value.
		Rotation value.	Reduction value.	
		per cent	per cent	
20 cc. expressed muscle juice from rabbit muscle + 30 units insulin + 100 cc. 4 per cent $\alpha$ - $\beta$ -glucose + 100 cc. 0.9 per cent NaCl solution.	2.66	0.875	0.878	+52.3°
15 cc. expressed muscle juice from rat muscle + 20 units insulin + 100 cc. 4 per cent $\alpha$ - $\beta$ -glucose + 10 cc. 0.9 per cent NaCl solution.	2.19	0.720	0.711	+53.2°

It follows therefore that the heating had destroyed or at any rate inactivated the insulin complement. Thus with respect to thermostability also, insulin complement is distinct from Meyerhof's muscle coferment.

*D. Is Insulin Complement Combined with the Intact Cell Stroma or Does It Occur in the Tissue Fluids?*

The muscle juice was expressed from rabbit and rat muscle according to the directions given by Buchner.

The preparation of the expressed muscle juice was made through

the kindness of Cand. pharm. Gad-Andresen at the Medicinal Compagni's laboratory. The whole process from the time the animal was killed till the expressed juice was prepared lasted 20 minutes. Directly afterwards the juice was mixed with a glucose solution containing insulin. The details of the experiment will be seen in Table IV. The mixture was shaken for 2 hours at 37°C. and then a sample was taken for dialysis which lasted half an hour, after which the glucose concentration of the dialysate was determined by the reduction and rotation values. From the results in Table IV it will be observed that both the experiments

TABLE V.

*Action of Rabbit Muscle Kept for 20 Minutes and Insulin on  $\alpha$ - $\beta$ -Glucose in Vitro.*

Conditions of experiment.	Ventate degree directly observed.	Glucose.		Specific rotation value.	Ventate degree directly observed.	Glucose.		Specific rotation value.
		Rotation.	Reduction.			Rotation value.	Reduction value.	
		per cent	per cent			per cent	per cent	
15gm. rabbit muscle kept for 20 min. Then 20 units insulin + 100 cc. 2 per cent $\alpha$ - $\beta$ -glucose + 100 cc. 0.9 per cent NaCl solution.	1.17	0.383	0.492	+38.5°	1.50°	0.492	0.492	+52.5°

gave similar results. The rotation and reduction values were found to be identical, so that no change in the  $\alpha$ - $\beta$ -glucose could be demonstrated.

In our earlier experiments with muscle tissue we always added the muscle to the glucose and insulin solution in the course of a few minutes after the animal's death. As stated, however, 20 minutes elapsed before the preparation of the expressed juice was finished. It was, therefore, necessary that we should make a control experiment with rabbit muscle which was not used until it had been kept for 20 minutes. The results of this experiment are recorded in Table V and show that after 20 minutes there was

enough active insulin complement in the muscle to form new-glucose from the  $\alpha$ - $\beta$ -glucose.

The fact that we cannot change  $\alpha$ - $\beta$ -glucose with the expressed juice in conjunction with insulin can only mean that insulin complement is not present in the juice as prepared by Buchner's directions, but must be assumed to be united with the intact cell stroma.

#### RÉSUMÉ.

1. Insulin complement is not identical with the muscle co-ferment demonstrated by Meyerhof in 1918.

2. Insulin complement is not removed from muscle by washing with water.

3. Insulin complement will not tolerate heating to 70°C. for 2 minutes.

4. Insulin complement cannot be detected in the expressed juice of muscle (prepared by Buchner's method), but must be assumed to be combined with the intact cell stroma.

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## NOTE. THE RECRYSTALLIZATION OF UREASE.

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(Received for publication, June 30, 1926.)

In a preceding paper<sup>1</sup> it was stated that attempts to recrystallize urease were not entirely successful. The fact that few or no crystals could be obtained by chilling and seeding concentrated solutions of the octahedral crystals to which acetone had been added led me to consider what conditions might have been the cause of the crystallization from the original extracts.

It was believed that the reaction of the solution, or the presence of certain organic salts, might have been responsible. Accordingly, the pH of the original extracts was determined by the indicator method. This was found to be 6.1. It will be interesting to learn whether this reaction is in the neighborhood of the isoelectric point of urease, as I am inclined to believe. A phosphate solution of pH 6.1 was prepared by adding 14.3 cc. of 0.1 M NaOH to 50 cc. of 0.2 M  $\text{KH}_2\text{PO}_4$  and to this was added enough acetone to make the acetone concentration 32 per cent. The crystals obtained from 100 gm. of jack bean meal were dissolved in 3.5 cc. of water, centrifuged from insoluble matter, mixed with 1.5 cc. of acetone, and cooled in the ice chest. To this enzyme solution I added the chilled phosphate buffer. A fine precipitate soon settled out, but it was not possible to say whether or not the material was crystalline. I repeated the experiment, adding the buffer a few drops at a time and only using 25 drops in all. This time a good crop of octahedral crystals was obtained. It was found that a buffer solution of pH 6.3 could also be used.

Analyses made on several lots of the recrystallized urease for enzyme activity and for total nitrogen have shown that on the

<sup>1</sup> Sumner, J. B., *J. Biol. Chem.*, 1926, lxi, 435.



basis of nitrogen content and within the limit of error of the analysis *recrystallized urease has the same activity as once crystallized urease*. This is the last and best evidence that the octahedral crystals are indeed identical with urease. I have been able to recrystallize urease twice, with a total loss of about 80 per cent of the enzyme taken at the start. If enough material were used I believe that one could recrystallize as many times as might be desired.

I wish to call attention to a point that I should have mentioned in my previous paper;<sup>1</sup> namely, that when testing solutions of crystallized urease I have been able to avoid the inactivation that is caused by dilution with water by using a drop of concentrated urease solution instead of 1 cc. of dilute urease solution. The volume of the drop of urease has been measured by counting the number of drops delivered by the pipette. The drop has been allowed to fall into a mixture of 3 cc. of urea-phosphate solution and 3 cc. of water and after the action an aliquot has been Nesslerized. This method is not very accurate and I hope to improve upon it before long.

The urease activity of "Arlco" jack bean meal has been found to be 150 units per gm. This would make the content of urease 0.15 per cent. One sample of jack bean meal ground in our laboratory showed 108 units per gm.; while another showed 140 units per gm. The Arlco meal may be richer in urease because of the fact that it has been defatted, dried, and possibly freed from the inactive hulls.

I have investigated the effect of temperature upon the extraction of urease from jack bean meal with dilute acetone and find that if ice cold 31.6 per cent acetone is used the precipitate of crystals that is obtained contains a very considerable amount of insoluble matter that apparently consists either of canavalin or of some unknown jack bean protein. On the contrary, when the dilute acetone is added at a temperature of about 28°C. this insoluble impurity is largely absent. The reason seems to be that at an elevated temperature the material in question combines with acids that are present in the extract and is precipitated before it has opportunity to pass through the filter. Accordingly it is best to use the diluted acetone at a temperature of about 28°C. for preparing the crystals.

## AMINO ACID CATABOLISM.

### I. THE FATE OF $\gamma$ -AMINO BUTYRIC ACID AND $\delta$ -AMINO VALERIC ACID IN THE PHLORHIZINIZED DOG.\*

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(Received for publication, June 19, 1926.)

#### INTRODUCTION.

. One of the important and interesting problems of intermediary metabolism is that of the degradation of the amino acids. While the catabolism of the simple  $\alpha$ -amino acids has been rather carefully investigated, little is known of the fate of a number of the other products resulting from the complete hydrolysis of protein. As Dakin (1) has indicated, the catabolic fate of the diamino acids is obscure. There is considerable evidence that  $\alpha$ -amino acids may be converted into fatty acids having 1 less carbon atom, through the intermediate stage of an  $\alpha$ -keto acid. The question arises as to whether or not the degradation of the diamino and the dicarboxylic acids does not start by the same route taken by the simple monoamino, monocarboxylic acids. Indeed unless there is an increase in the stability of the  $\alpha$ -amino group, this would be expected to be the first point of attack not only in the simple  $\alpha$ -amino acids but in the other naturally occurring amino acids as well.

Corley and Rose (2) have recently published results that are suggestive of one possible fate of glutamic acid.  $\alpha$ -Keto glutaric acid was shown to be transformed to sugar in the diabetic dog to the same extent as glutamic and succinic acids. This can be interpreted as indicating the possibility of glutamic acid being converted successively to  $\alpha$ -keto glutaric and succinic acids in the

\* Assisted by a grant from the David Trautman Schwartz research fund.

course of its destruction in the body. Should the diamino acids have an analogous path of oxidation, they would form the  $\alpha$ -keto acids and the corresponding  $\omega$ -amino acids, having 1 less carbon atom, ornithine yielding  $\gamma$ -amino butyric acid, and lysine,  $\delta$ -amino valeric acid. There are several reactions that have been conducted *in vitro* that are of interest in this connection. The evidence of Dunn and Schmidt (3) that the reactivity of amino groups with nitrous acid varies with the distance from the carboxyl group, suggests that in the body as well the  $\alpha$ -amino group may be the most labile. In addition, Neuberg (4) has shown that under the action of hydrogen peroxide in the presence of ferrous sulfate,  $\alpha$ - $\beta$ -diamino propionic acid is converted to  $\alpha$ -amino acetaldehyde. To substantiate the belief that it is the  $\alpha$ -amino group that is removed, there are the additional observations that, under similar conditions of oxidation, serine yields  $\alpha$ -hydroxy acetaldehyde while isoserine yields  $\alpha$ -amino acetaldehyde.

In seeming opposition to the conception of an oxidative deamination of certain of these amino acids, there are the reports that during putrefaction, through reductive processes, glutamic acid (5) gives rise to  $\gamma$ -amino butyric acid, arginine (5) and proline (6) to  $\delta$ -amino valeric acid, and lysine (5) to  $\epsilon$ -amino caproic acid. A very fruitful source of information in regard to intermediary metabolism has been through experimental diabetes. A study of the fate of certain of the  $\omega$ -amino acids in the diabetic organism should be of value, because of their bearing on amino acid catabolism and also on the more general problem of the removal of amino groups in other than the  $\alpha$  position.

$\gamma$ -Amino butyric acid and  $\delta$ -amino valeric acid have been synthesized and their fate in the completely phlorhizinized dog determined.

#### EXPERIMENTAL.

##### *Synthesis of $\gamma$ -Amino Butyric Acid.*

According to the method of Gabriel (7) trimethylene chlorobromide was converted to  $\gamma$ -chlorobutyronitrile, which was condensed with potassium phthalimide to form  $\gamma$ -cyanopropylphthalimide, which yielded the desired compound upon hydrolysis with sulfuric acid. After repeated recrystallization from methyl

alcohol and ether, the substance melted at 183–184°C. This is the melting point given by Gabriel, but others have reported somewhat higher figures as 193°C. (8), or 202°C. (9).

The trimethylene chlorobromide was prepared by two methods; from trimethylene chlorohydrin by treatment with constant boiling hydrobromic acid (10) or from allyl alcohol prepared from glycerol and oxalic acid (11). Allyl chloride prepared through the use of phosphorus trichloride (12) absorbed hydrobromic acid in the sunlight to form trimethylene chlorobromide (13).

#### *Synthesis of $\delta$ -Amino Valeric Acid.*

According to the method of Gabriel and collaborators (14, 15),  $\gamma$ -bromopropylphthalimide, obtained by treating potassium phthalimide with trimethylene bromide, was condensed with malonic ester in the presence of sodium ethylate. Instead of hydrolyzing the resulting phthalimidopropyl malonic ester under pressure, it was boiled vigorously with a free flame with 6 parts of concentrated hydrochloric acid for 12 hours. Some difficulty was encountered in crystallizing this acid, since ether precipitated it as a syrup from a solution in methyl alcohol. After repeated reprecipitations, the acid was obtained as a hard solid with a melting point of 153–154°C. (theoretical 154–156°C.).

#### *Experimental Procedure.*

The experimental procedure was that described by Corley and Rose (2).

#### *Results.*

Typical experiments for  $\gamma$ -amino butyric acid are given in Tables I and II, and for  $\delta$ -amino valeric acid in Tables III and IV. Tables V and VI summarize the results obtained with both acids. As reported by Schotten (16, 17) both acids seem to be without toxic effects.

In calculating the extra glucose excretion, we have subtracted from the net G:N ratio (*i.e.* the ratio obtained after correcting for the nitrogen in the compound injected) of the day the acid was administered, the average of the 2 preceding days and the 2 following days, and then multiplied the resulting figure by the

TABLE I.

*Sugar Formation from  $\gamma$ -Amino Butyric Acid.*

Dog 6, weight 8.2 kilos.

Date.	Acid administered.	Urine.				Blood non-protein nitrogen.	Extra glucose.			
		Volume.	Glucose.	Total nitrogen.	G:N		Theoretical.		Found.	
							For 3 carbons.	For 2 carbons.	1st method of calculation.	2nd method of calculation.
	gm.	cc.	gm.	gm.		mg.	gm.	gm.	gm.	gm.
Apr. 14*		400	19.1	6.69	2.86	34.2				
" 15		600	28.5	9.56	2.98					
" 16	4.0†	500	27.4	8.94	3.07		3.49	2.33	3.61	3.93
" 17		400	21.0	7.33	2.86	39.5				
" 18		400	16.3	6.24	2.61					

\* Animal began fasting April 1. From April 13 the animal received 0.8 gm. of phlorhizin every day.

† Acid injected in 20 per cent aqueous solution in two equal portions at 6.00 p.m. and 8.00 p.m. 4.0 gm. of the acid contain 0.543 gm. of nitrogen.

TABLE II.

*Sugar Formation from  $\gamma$ -Amino Butyric Acid.*

Dog 9, weight 11.5 kilos.

Date.	Acid administered.	Urine.				Blood non-protein nitrogen.	Extra glucose.			
		Volume.	Glucose.	Total nitrogen.	G:N		Theoretical.		Found.	
							For 3 carbons.	For 2 carbons.	1st method of calculation.	2nd method of calculation.
	gm.	cc.	gm.	gm.		mg.	gm.	gm.	gm.	gm.
May 9*		500	15.3	4.80	3.18	39.8				
" 10		500	14.1	4.80	2.94					
" 11		500	15.4	4.80	3.21					
" 12	5.0†	500	16.2	5.03	3.22		4.36	2.92	2.57	4.38
" 13		900	17.4	5.17	3.35	34.2				
" 14		1200	13.2	4.34	3.04					

\* Started fasting May 6, and from that time received 1.0 gm. of phlorhizin daily.

† Acid injected in 20 per cent aqueous solution. 3.0 gm. at 12.30 p.m. and 2 gm. at 4.30 p.m. 5.0 gm. of the acid contain 0.68 gm. of nitrogen.

TABLE III.

*Sugar Formation from  $\delta$ -Amino Valeric Acid.*

Dog 7, weight 15 kilos.

Date.	Acid administered.	Urine.				Blood non-protein nitrogen.	Extra glucose.			
		Volume.	Glucose.	Total nitrogen.	G:N		Theoretical.		Found.	
							For 3 carbons.	For 2 carbons.	1st method of calculation.	2nd method of calculation.
	gm.	cc.	gm.	gm.		mg.	gm.	gm.	gm.	gm.
Apr. 16*		1500	50.72	16.80	3.02	23.9				
" 17		1500	32.56	10.89	2.99					
" 18	4.0†	1200	31.60	10.99	2.87		3.07	2.05	0.73	0.0
" 19		1200	27.52	9.42	2.92	28.9				
" 20		1000	24.32	7.95	3.06					

\* Animal fasted from April 11, and given 1.2 gm. of phlorhizin daily from April 12.

† Acid injected in 20 per cent aqueous solution, in two equal portions at 5.00 p.m. and 8.00 p.m. 4.0 gm. of the acid contain 0.48 gm. of nitrogen.

TABLE IV.

*Sugar Formation from  $\delta$ -Amino Valeric Acid.*

Dog 10, weight 10.5 kilos.

Date.	Acid administered.	Urine.				Blood non-protein nitrogen.	Extra glucose.			
		Volume.	Glucose.	Total nitrogen.	G:N		Theoretical.		Found.	
							For 3 carbons.	For 2 carbons.	1st method of calculation.	2nd method of calculation.
	gm.	cc.	gm.	gm.		mg.	gm.	gm.	gm.	gm.
Apr. 30*		300	26.1	7.98	3.27	38.9				
May 1		1400	32.4	9.67	3.35					
" 2	7.0†	900	39.0	12.04	3.24		5.88	3.59	2.69	0.55
" 3		1300	21.6	6.96	3.10	44.6				
" 4		1300	25.6	7.75	3.30					

\* Animal fasted from April 16, and treated daily with 1.0 gm. of phlorhizin from April 28.

† Acid injected in 20 per cent aqueous solution, 2.5 gm. at 1.00 p.m., 2.5 gm. at 6.30 p.m., 2.0 gm. at 10.30 p.m. 7.0 gm. of the acid contain 0.84 gm. of nitrogen.

nitrogen excretion of the experimental day. The assumption must be made, and it seems scarcely unwarranted, that the nitrogen of the substances administered will be excreted in the urine. The first calculations were made on the basis that the nitrogen and the extra glucose would appear within a few hours. It was

TABLE V.

Summary.

*Extra Glucose Formation from  $\gamma$ -Amino Butyric Acid.*

Dog No.	Acid administered.	Theoretical.		Found.	
		If 3 carbons converted.	If 2 carbons converted.	1st method of calculation.	2nd method of calculation.
	gm.	gm.	gm.	gm.	gm.
2	4.0	3.49	2.33	2.54	2.42
6	4.0	3.49	2.33	3.61	3.93
9	5.0	4.36	2.92	2.57	4.38
13	6.5	5.68	3.79	3.26	4.88
Total.	19.5	17.05	11.37	11.98	15.61

TABLE VI.

Summary.

*Extra Glucose Formation from  $\delta$ -Amino Valeric Acid.*

Dog No.	Acid administered.	Theoretical.		Found.	
		If 3 carbons converted.	If 2 carbons converted.	1st method of calculation.	2nd method of calculation.
	gm.	gm.	gm.	gm.	gm.
7	4.0	3.07	2.05	0.73	0.0
10	7.0	5.38	3.59	2.69	0.55
11	7.0	5.38	3.59	1.78	0.0
12	5.0	3.84	2.57	0.0	0.0
Total.	23.0	17.67	11.80	5.20	0.55

noted in a number of cases, particularly those in which larger amounts of the compounds were injected, that the G:N ratio showed alterations that could be most readily explained if it were considered that the appearance of the catabolites in the urine was slightly delayed. For example, in the case of Dog 9, Table II, the

G:N ratio of the day following the administration of the acid as well as the experimental day is distinctly higher than those before or after. Similarly, as shown in Table IV, the G:N ratio of May 3 is distinctly lower than the general level, as if the nitrogen excretion were somewhat delayed. Because of these reasons, and in view of the fact that the procedure would seem to have no mathematical objection, there also has been employed a second method of calculation that is felt to give more reliable results. The G:N ratio of the experimental day and the following day is determined by dividing the combined sugar excretion by the combined nitrogen excretion minus the nitrogen in the acid administered. The difference between this figure and the average of the 2 preceding days and the 2nd day following, times the net combined nitrogen excretion for the 2 days, gives the extra sugar formation. If sugar and nitrogen derived from the administered substances are eliminated immediately, this method should give figures similar to those obtained by the first method of calculation.

#### DISCUSSION.

The results are interpreted to indicate that  $\gamma$ -amino butyric acid is a sugar former, while  $\delta$ -amino valeric acid is not. 23 gm. of  $\delta$ -amino valeric acid, which would yield 11.8 gm. or 17.67 gm. if 2 or 3 carbon atoms respectively were converted to glucose, caused an apparent sugar excretion of 5.2 gm. by one method of calculation, or 0.55 gm. by the other method. Neither of these figures is felt to indicate actual glucose formation from the administered compound. In general, the possible catabolic paths of this acid would seem to be either reductive deamination or oxidation of any one of the carbon atoms. Oxidation of the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, or  $\delta$ -carbon atoms would probably give  $\gamma$ -amino butyric acid,  $\beta$ -alanine, succinic acid, or glutaric acid respectively, while reductive deamination would form valeric acid. Since  $\gamma$ -amino butyric acid, succinic acid (18), and valeric acid (19) are converted to sugar in the diabetic organism, of the substances suggested it is only  $\beta$ -alanine and glutaric acid that need be considered as possible intermediates in the degradation of  $\delta$ -amino valeric acid. The fate of the former is unknown, but it can be investigated profitably. Ringer, Frankel, and Jonas (18) have suggested that the oxidation



of the diamino acids proceeds in such a way as to give a dicarboxylic acid having 1 less carbon atom, lysine and ornithine giving glutaric and succinic acids respectively. While there is little direct evidence as to the fact or mechanism of such a reaction, it is one that merits careful consideration.

The possible paths of degradation of  $\gamma$ -amino butyric acid are the same as those of  $\delta$ -amino valeric acid. Reductive deamination would give butyric acid;  $\alpha$ -oxidation,  $\beta$ -alanine;  $\beta$ -oxidation, glycine or possibly malonic acid; while  $\gamma$ -oxidation would yield succinic acid. Ringer, Frankel, and Jonas (18) suggested that malonic acid may be a sugar former, but as Dakin (1) has pointed out, their results are not convincing. Butyric acid is not a sugar former,  $\beta$ -alanine may or may not be, while glycine and succinic acid yield 2 and 3 carbon atoms respectively as glucose. Inasmuch as the analogous reactions of  $\delta$ -amino valeric acid would seem to be precluded, since they would yield the sugar-forming  $\gamma$ -amino butyric acid and succinic acid, the formation of  $\beta$ -alanine or malonic acid as intermediary metabolites is contraindicated.

19.5 gm. of  $\gamma$ -amino butyric acid yielded 11.98 gm. of extra glucose by the first method of calculation and 15.6 gm. by the second. The theoretical amounts would be 11.35 gm. if 2 carbon atoms were transformed to sugar, and 17.05 gm. if 3 were so converted. While it is not to be denied that the results might be interpreted as indicating that only 2 carbons of  $\gamma$ -amino butyric acid are synthesized to form glucose, since it is believed that the second method of calculation leads to more reliable results, it is felt that these experiments are to be taken as indicating that three-fourths of the carbon of this amino acid is converted to glucose.

While it thus seems that the conversion of  $\gamma$ -amino butyric acid to succinic acid is a possible path of oxidation, the mechanism of such a reaction leaves much to be explained. Mayer (20) has shown that glyceric acid appears in the urine when  $\alpha$ - $\beta$ -diamino propionic acid is injected, showing that the animal body has the power of replacing with a hydroxyl group an amino group in other than the  $\alpha$ -position. Furthermore, alcohols appear to undergo oxidation in the body with the formation of the corresponding acids (1, 21). It is also suggestive that barium permanganate oxidizes arginine to succinic acid (22).

Thomas and Goerne (23) report that  $\epsilon$ -amino caproic acid is

toxic but that when administered it is apparently completely burned. They consider that  $\gamma$ -amino butyric acid may be an intermediate catabolite, but offer no evidence to substantiate their belief.

The close structural relations between ornithine, proline, and glutamic acid (24), all of which yield three-fifths of their carbon as glucose, suggest that their catabolic path may be similar. A reductive splitting of the ring of proline as observed by Neuberg (6) in studies on putrefactive changes, gives  $\delta$ -amino valeric acid. The possibility arises therefore that during the catabolic rupture of the ring, there is a loss of 1 carbon atom giving  $\gamma$ -amino butyric acid, a sugar former.

The fate of a number of compounds related to  $\gamma$ -amino butyric acid and  $\delta$ -amino valeric acid should be of interest and value and will be reported in subsequent papers.

#### SUMMARY.

1.  $\delta$ -Amino valeric acid is not a sugar former in the phlorhizinized dog.

2.  $\gamma$ -Amino butyric acid is a sugar former and is believed to yield 3 of its carbon atoms as glucose in the phlorhizinized dog.

3. It is suggested that one of the paths of catabolism of the diamino acids is through the stage of the acids having 1 less carbon atom and with an amino group in the terminal position.

4. It is suggested that one of the paths of catabolism of the amino acids with the amino group in the terminal position may be through the stage of the corresponding dicarboxylic acids.

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# THE PREPARATION AND PROPERTIES OF EPHEDRINE AND ITS SALTS.

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## PLATE 1.

With the development in scientific medicine of the clinical use of the alkaloid ephedrine, there has been an increasing interest in the preparation of this alkaloid, and an immediate need for accurate data in the standardization of its salts.

In these laboratories large amounts of ephedrine have been prepared and it has been found that the basic substance isolated from the Chinese drug Ma Huang, variously identified as *Ephedra vulgaris* Rich. var. *helvetica* Hk. et Thoms (1) and *Ephedra equisetina*, Bge., (2) consists chiefly of ephedrine together with about 20 per cent of its isomer pseudoephedrine. These two alkaloids were first prepared by Nagai (3) and Merck (4) respectively. They were found to be mutually convertible by boiling with hydrochloric acid and in other ways (5), and have the empirical formula  $C_{10}H_{15}ON$ .

The chemical structure of ephedrine, and its isomer  $OH \cdot CH \cdot C_6H_5 \cdot CH \cdot CH_3NH \cdot CH_3$ , has been studied by many workers (6-8) and synthetically proven for both alkaloids (9-12). It shows a close relationship in chemical structure to epinephrine, to which it has been found to have similar, and in some respects superior physiological effects (13-16).

The separation of the two alkaloids by some clear cut method has been the object of this investigation. While the salts of ephedrine are better crystallized and less soluble in water and alcohol than those of pseudoephedrine it has been found that the best means of separation depends upon the striking difference in the solubility of their oxalates in cold water.

Pure preparations of the alkaloids and their salts were made and studied. While some of the physical constants of the salts obtained do not agree with those published by Chen (17), they do confirm such as are published by several other workers (18). The pure base heretofore considered to be levorotatory was found to be dextrorotatory in water, and levorotatory in alcohol.

#### EXPERIMENTAL.

*Extraction.*—1 kilo of powdered Ma Huang was extracted with cold benzene in the presence of dilute  $\text{Na}_2\text{CO}_3$  solution, and the benzene extract was shaken up with a sufficient quantity of dilute  $\text{HCl}$  to remove the basic substances. The acid solution was made alkaline with solid  $\text{K}_2\text{CO}_3$  and the liberated base was then extracted with chloroform. The chloroform solution, when dried over anhydrous  $\text{Na}_2\text{SO}_4$  and distilled, gave 2.6 gm. of crude base.

*Preparation of Ephedrine HCl by Fractional Crystallization.*—The crude base obtained as above was taken up with about twice its weight of alcohol and neutralized with concentrated  $\text{HCl}$  diluted with twice its volume of alcohol. Nearly pure ephedrine hydrochloride crystallized out on standing. After filtering it was washed with a mixture of alcohol and ether, and then with pure ether, and dried. A further quantity of ephedrine hydrochloride may be got by concentrating the mother liquors and washings. The final mother liquor was kept for the isolation of pseudoephedrine (see below).

Ephedrine hydrochloride crystallized out from alcohol in prismatic needles, m.p.  $216^\circ\text{C}.$ ,  $[\alpha]_D^{22} -32.5^\circ$  (see Fig. 1).

The salts prepared by fractional crystallization show no change in the melting point when recrystallized seven times. In many of our experiments the salts were recrystallized twelve times.

*Separation of the Oxalates by Difference of Solubility.*—The crude base as obtained when treated with oxalic acid gave a clear cut separation of the two alkaloidal salts. Ephedrine oxalate being only very slightly soluble in cold water and pseudoephedrine oxalate being exceedingly soluble, the soluble and insoluble products represented a separation of the two alkaloids.

*Preparation of Pure Ephedrine Base.*—5 gm. of pure ephedrine hydrochloride, dissolved in a sufficient quantity of water, were made alkaline with solid  $\text{K}_2\text{CO}_3$  until two layers were formed and

extracted twice with chloroform. The chloroform solution was well dried over anhydrous  $\text{Na}_2\text{SO}_4$  and distilled. On cooling the residue crystallized out in rhombic crystals. It was recrystallized by dissolving in a small quantity of alcohol and then adding a sufficient quantity of petroleum ether, m.p.  $43^\circ\text{C}$ . (see Fig. 2).

*Properties of Pure Ephedrine.*—Ephedrine was found to be very soluble in water, alcohol, and chloroform and nearly insoluble in petroleum ether upon cooling. Being a strong base, ephedrine displaces ammonia from its salts. Solutions of the salts in water varying from 1 to 10 per cent were found to be exceedingly stable. No change in strength occurred after 6 months storage at room temperature. The solutions are quite stable at boiling temperature. Its specific rotation varied with the different solvents used.

1.  $[\alpha]_D^{25} + 13.75^\circ$  in water.
2.  $[\alpha]_D^{25} - 5.5^\circ$  in absolute alcohol.

A part of the pure ephedrine so obtained was transformed into its corresponding salts. They gave the following physical constants.

*Ephedrine Hydrochloride.*  $\text{C}_{10}\text{H}_{15}\text{ON} \cdot \text{HCl}$ .—Prismatic needles; m.p.  $216^\circ\text{C}$ .,  $[\alpha]_D^{22} - 32.5^\circ$ . Easily soluble in alcohol and water. Its aqueous solution is stable at boiling temperature.

*Ephedrine Sulfate.*  $\text{C}_{10}\text{H}_{15}\text{ON} \frac{1}{2} \text{H}_2\text{SO}_4$ .—Hexagonal plates; m.p.  $257^\circ\text{C}$ .,  $[\alpha]_D^{22} - 30^\circ$ . Difficultly soluble in alcohol, easily soluble in water, neutral to litmus.

*Analysis.* 0.2 gm. substance gave 0.109 gm.  $\text{BaSO}_4$ .

S = 7.48 per cent (theoretical 7.47 per cent).

*Ephedrine Oxalate.*  $2\text{C}_{10}\text{H}_{15}\text{ON} \cdot \text{C}_2\text{H}_2\text{O}_4$ .—Prismatic needles from water; m.p.  $245^\circ\text{C}$ . with decomposition; neutral to litmus; only very slightly soluble in cold water (see Fig. 3).

*Ephedrine Phosphate.*  $\text{C}_{10}\text{H}_{15}\text{ON} \cdot \text{H}_3\text{PO}_4$ .—Crystallized from alcohol in long silky needles; m.p.  $178^\circ\text{C}$ .; acid to litmus.

*Analysis.* 0.1 gm. substance gave 0.0412 gm.  $\text{Mg}_3\text{P}_2\text{O}_7$ .

P = 11.5 per cent (theoretical = 11.7 per cent).

### *Pseudoephedrine.*

The alcoholic mother liquor obtained after the removal of ephedrine hydrochloride as indicated above was first concentrated

and then evaporated to dryness on a water bath. The residue was taken up with a little water, made alkaline with  $K_2CO_3$ , extracted with chloroform, and distilled. The basic residue was transformed into its corresponding sulfate by neutralizing with concentrated sulfuric acid diluted previously with alcohol. The crude sulfate so obtained consisted chiefly of pseudoephedrine sulfate. Its free base was obtained by dissolving in water, making alkaline with  $K_2CO_3$ , and extracting with chloroform. Pure pseudoephedrine crystallized out from alcohol in rhombic prisms, m.p.  $118^\circ C.$ ,  $[\alpha]_D^{22} + 50^\circ$  (see Fig. 4). Unlike ephedrine it was only slightly soluble in water. Its salts were prepared and gave the following physical constants.

*Pseudoephedrine Hydrochloride.*  $C_{10}H_{15}ON \cdot HCl$ .—Crystallized from alcohol in stout needles; m.p.  $179-181^\circ C.$ ,  $[\alpha]_D^{22} + 58.75^\circ$ ; very soluble in water and in alcohol (see Fig. 5).

*Pseudoephedrine Sulfate.*  $C_{10}H_{15}ON \frac{1}{2} H_2SO_4$ .—Prismatic needles; no sharp m.p.;  $[\alpha]_D^{22} + 52.5^\circ$ ; easily soluble in water and in alcohol.

*Pseudoephedrine Oxalate.*  $2C_{10}H_{15}ON \cdot C_2H_2O_4$ .—Needles; m.p.  $218^\circ$  with decomposition; difficultly soluble in alcohol; very soluble in cold  $H_2O$ ; neutral to litmus (see Fig. 6).

*Action of HCl upon Ephedrine. Preparation of Pseudoephedrine.*

50 gm. ephedrine HCl.  
350 cc. concentrated HCl.  
175 cc.  $H_2O$ .

Boiled gently with reflux condenser for 12 hours. The acid solution was evaporated low over a water bath and allowed to crystallize; the crystallized HCl salt consisted of nearly pure unchanged ephedrine hydrochloride. If the acid solution had been sufficiently concentrated practically all the unchanged ephedrine hydrochloride would have crystallized out. Filtered, washed with a little dilute HCl, and used the filtrate for the preparation of pseudoephedrine. The acid filtrate was then evaporated to dryness over a water bath. The residue was taken up with a little water, made alkaline with  $K_2CO_3$ , and extracted with chloroform. The chloroform solution, when well dried over anhydrous  $Na_2SO_4$  and concentrated, deposited pseudoephedrine in rhombic crystals. The base obtained melted at  $118^\circ C.$  and was identical in all respects with pseudoephedrine obtained from the crude drug.

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#### EXPLANATION OF PLATE 1.

FIG. 1. Ephedrine hydrochloride. M.p.  $216^{\circ}\text{C}.$ ,  $[\alpha]_{\text{D}}^{25} - 32.5^{\circ}$ .

FIG. 2. Ephedrine. M.p.  $43^{\circ}\text{C}.$ ,  $[\alpha]_{\text{D}}^{25} + 13.75^{\circ}$  in water.  $[\alpha]_{\text{D}}^{25} - 5.5^{\circ}$  in absolute alcohol.

FIG. 3. Ephedrine oxalate. M.p.  $245^{\circ}\text{C}.$

FIG. 4. Pseudoephedrine. M.p.  $118^{\circ}\text{C}.$ ,  $[\alpha]_{\text{D}}^{25} + 50^{\circ}$  in alcohol.

FIG. 5. Pseudoephedrine hydrochloride. M.p.  $179^{\circ}\text{C}.$ ,  $[\alpha]_{\text{D}}^{25} + 58.75^{\circ}$ .

FIG. 6. Pseudoephedrine oxalate. M.p.  $218^{\circ}\text{C}.$

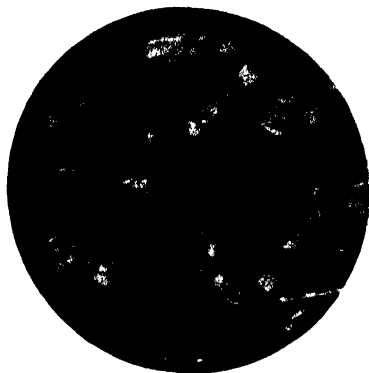


FIG. 1.



FIG. 2.

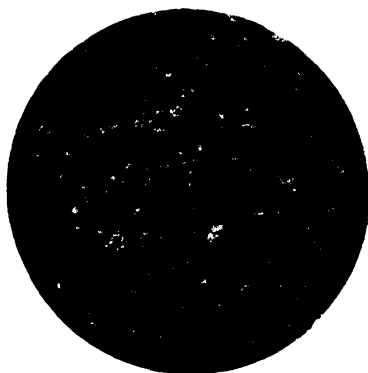


FIG. 3.

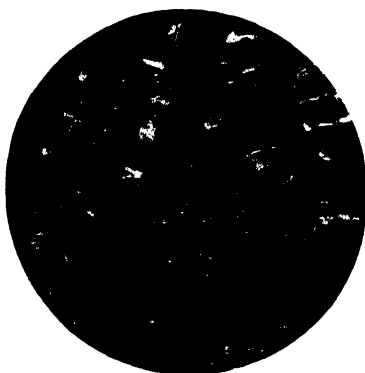


FIG. 4.

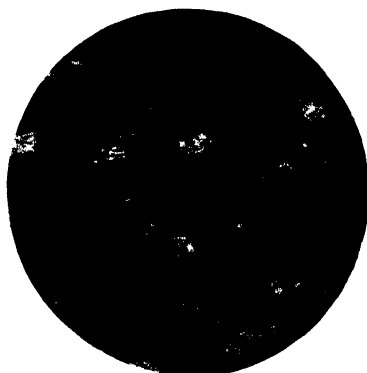


FIG. 5.

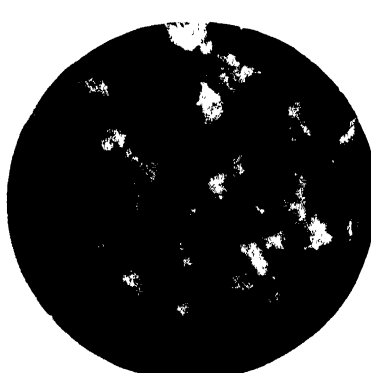


FIG. 6.

(Chou: Ephedrine.)



## A GAS ANALYSIS APPARATUS MODIFIED FOR THE DETERMINATION OF METHANE IN METABOLISM EXPERIMENTS.

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The metabolism of certain types of animals, particularly ruminants, is accompanied by the alimentary production of methane. The amount of this material given off into the air of a respiration chamber may be determined by passing a given volume of air, which has been freed of carbon dioxide and water, through a combustion furnace as was done at the Institute of Animal Nutrition in Pennsylvania.<sup>1</sup> It may also be determined gasometrically by means of a gas analysis apparatus adapted for the determination as has been done by Klein<sup>2</sup> and by Möllgaard.<sup>3</sup> In the metabolism studies with ruminants at the University of New Hampshire Experiment Station, a gas analysis apparatus devised in this Laboratory<sup>4</sup> has been in use for several years for the determination of carbon dioxide and oxygen in the outgoing air from the chamber. This apparatus has now been modified in such a way that the added determination of methane may be carried out with approximately the same degree of accuracy as is obtained in the other analyses of chamber air and outdoor air. A sketch of the modified apparatus is shown in Fig. 1. The major portion of this apparatus remains as originally described, which, briefly, is constructed on the Haldane principle with the burette so modified that carbon dioxide may be determined up to 1.7 per cent and oxygen values

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<sup>3</sup> Möllgaard, H., and Andersen, A. C., *Den Kongelige Veterinaer-og Landbohøjskole Aarskrift*, København, 1917, 238.

<sup>4</sup> Carpenter, T. M., *J. Metabol. Research*, 1923, iv, 1.

may be obtained corresponding to a deficit as great as 2 per cent. Readings are made to practically 0.001 per cent and the average accuracy in results obtained is a variation in several analyses of  $\pm 0.003$  per cent for carbon dioxide and  $\pm 0.005$  per cent for oxygen.

The arrangement shown in Fig. 1 is that of the original apparatus with the exception that inserted between the absorption pipettes, *D* and *E*, and the burette, *A*, is the three-way stop-cock, *R*, with connection to the combustion pipette, *Y*, for the determination of methane. The additional part is shown as *R*, *U*, *T*, *Y*, and *W*. Stop-cock *R* permits communication between the absorption pipettes *D* and *E* and the burette, or between the burette and the combustion pipette, *Y*. The lower end is left open so that the combustion wire may be inserted. Instead of a tight insertion in the bottom of the combustion pipette of the rubber stopper containing the glass tubes which carry the wire connections to the combustion coil, the stopper is enclosed in rubber tubing, connected to the bottom of the pipette, *Y*.<sup>5</sup> This rubber tubing is wired around the bottom of *Y* and around the top of the rubber stopper. This gives a good tight connection without danger of leakage and one that is not so rigid as would be obtained by the ordinary method in which the stopper is pushed into the bottom of the pipette. Provision for the rise of mercury from the pipette, *Y*, when gas is forced in for the combustion, is made in the reservoir, *U*. Stop-cock *T* is inserted for use in pressure test if such is necessary. The combustion wire consists of No. 33 Brown and Sharpe gauge platinum wire (0.18 mm. in diameter) 23 cm. in length, and is coiled in the form of a spiral, 2.5 mm. inside diameter and about 30 mm. in length, so that it may be inserted through the opening at the bottom of *Y*. This wire has a resistance of 1.9 ohms and in the analysis should be heated to dull redness when used with 110 volts and an external resistance in series of about 65 to 67 ohms causing a current of 1.5 amperes to flow. Platinum wires about No. 23 B. and S. in size (0.56 mm. in diameter) are

<sup>5</sup> For this detail in construction as well as for much information concerning the technique of metabolism experiments with ruminants I am indebted to Professor H. Möllgaard, Department of Animal Physiology, Royal Experiment Station, Copenhagen, whose laboratory I visited in February, 1925 (T.M.C.).

used for the connections to the combustion wire. The two wires are fused to the ends of the glass tubes and extend about 12 mm. into the tubes to the point where they are soldered to the copper wires of the remaining connections.

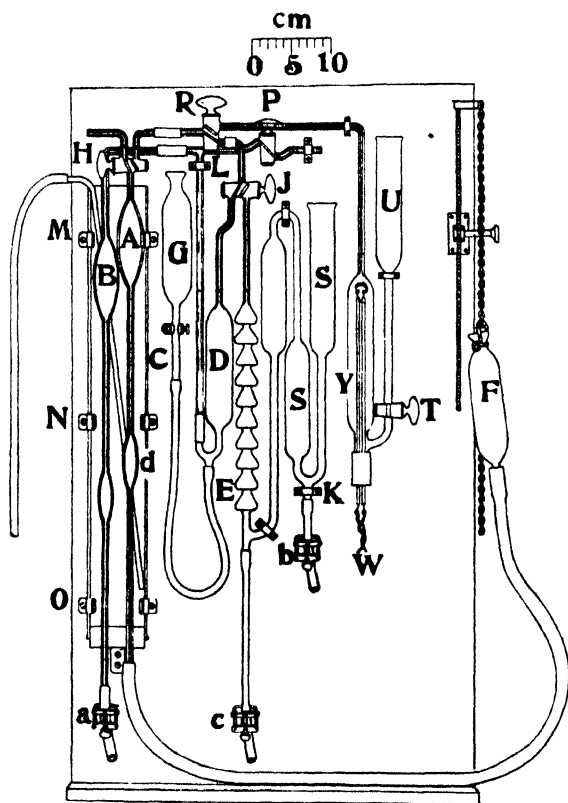


FIG. 1. A gas analysis apparatus for the determination of carbon dioxide, oxygen, and combustible gases (methane) in atmospheric and respiration chamber air. A, burette; B, compensator; D, pipette for absorption of carbon dioxide; E, pipette for absorption of oxygen; Y, pipette for combustible gases.

The analysis of gas containing methane is carried out as with all other types of apparatus used for the gasometric determinations of combustible gases. The gas is first freed from carbon dioxide, then passed into the combustion pipette where the combustible

gas is slowly burned, and again brought back for the absorption of carbon dioxide resulting from the combustion. This combustion of gas and absorption of carbon dioxide is repeated until the readings of the burette show that there is no more gas to be burned and the absorption of the carbon dioxide produced comes to an end. The first procedure in the use of the apparatus, however, is to demonstrate that, when the determination is carried out as, for example, with a gas containing methane, the readings are constant and that there is, therefore, no leak in the apparatus. If such an analysis is made in the usual way, it should be found that readings taken after the absorption of carbon dioxide will remain constant in spite of the fact that the determination is over a heated spiral. If this does not occur, there is a leak in some portion of the apparatus. The leak may be in the stop-cock, *R*, or, as our experience has shown, it may be around the fusion of the platinum wire and glass tubes in *Y*. In the latter case, the leak may be located by first putting the gas in *Y* under pressure with the glass tubes containing the platinum wire pulled down so that neither fusion nor tubes are exposed and then, if no leak is found, the gas may be pushed down to a point below the platinum spiral. If the leak is around the fusion, it will be shown by the diminishing size of the burette reading.

The accuracy of the determination of methane with this apparatus has been tested by analyzing mixtures of outdoor air and methane in which the quantities of methane are approximately those one would expect to find in the ventilating air from a chamber in which ruminants in full digestion are confined. The methane was produced from soda-lime and sodium acetate and when it was freely flowing, it was collected in tubes inverted in water. From one of these tubes it was transferred to an inverted burette, the top of which was connected to a three-way stop-cock for the admission of the gas. After the methane had been drawn into the burette, the two arms of the stop-cock were swept free of methane by means of outdoor air. One of the stop-cock openings was then connected to a tube leading into a Douglas bag and, by raising the mercury reservoir of the burette, the gas was delivered to the Douglas bag, readings for the volume of the gas being made at the beginning and end of the raising of the mercury in the burette. The three-way stop-cock was then reversed and the passage to the

bag was swept out with outdoor air measured through a meter. The passage having been sufficiently swept out, the outdoor air was continued through a larger tube and measured until the quantity we required for the mixture had been placed in the bag. The bag was then thoroughly kneaded and samples were withdrawn into mercury containers and stored. The percentage of methane was computed from the volumes of outdoor air and methane introduced into the Douglas bag. The gas was analyzed in the usual way.

The following procedure should give satisfactory results. The sample is drawn into the burette of the gas analysis apparatus and measured. As it is outdoor air mixed with methane, the carbon dioxide is first removed, in accordance with the routine given in the original description of the apparatus,<sup>4</sup> and the contraction measured in the usual manner. The remaining gas is then driven into the combustion pipette and the platinum ignition wire is heated to dull redness while the gas is being passed over it 10 times by raising and lowering the mercury leveling bulb (*F*) connected to the burette. Care should be taken, while this is being done, not to allow the mercury in the pipette to come in contact with the heated wire, since this would cause the wire to be short circuited and cooled and thus prolong the time of combustion. The gas is then passed 3 times over the potassium hydroxide in pipette *D* in order to absorb the carbon dioxide just produced. The operation is repeated by passing the gas 10 times over the hot wire, 3 times over the potassium hydroxide, and again 5 times over the hot wire, and 3 times over the potassium hydroxide. Next set, first, the mercury level in the combustion pipette, *Y*, and then the potassium hydroxide levels in *D* and read the burette. Repeat the preceding operation by passing the gas 5 times over the hot wire and 3 times over the potassium hydroxide, the determination being concluded when there is no decrease in the reading of the burette. One-third of the total contraction, which is the difference between the burette reading after the absorption of the carbon dioxide in the outdoor air of the sample and the final reading of the burette, represents the volume of methane in the sample. The percentage is readily computed. The gas should be allowed to cool following the combustion over the hot wire before any reading of the burette is made.



A sample determination, as made in the preliminary tests of this apparatus, is shown in the following analysis made on October 1, 1925.

TABLE I.  
*Results of Analyses of Mixtures of Methane and Outdoor Air.*

Date.	Analyst.	Methane found.	Methane in air mixture.*	Date.	Analyst.	Methane found.	Methane in air mixture.*
<i>1925</i>		<i>per cent</i>	<i>per cent</i>	<i>1925</i>		<i>per cent</i>	<i>per cent</i>
July 14	E.L.F.	0.211	0.209	Sept. 26	E.L.F.	0.302	
" 17	"	0.412	0.406	" 26	"	0.303	† 0.316†
" 17	"	0.221	0.210	" 29	M.H.V.	0.294	
" 20	"	0.229		" 29	"	0.282	† 0.316†
" 20	"	0.231	† 0.234	" 30	"	0.307	0.316†
" 21	"	0.230		Oct. 1	"	0.276	
" 21	"	0.228	† 0.254	" 1	"	0.280	
" 21	"	0.227		" 1	"	0.278	† 0.289
" 30	"	0.327		" 1	"	0.277	
" 30	"	0.326	† 0.316	Nov. 3	E.L.F.	0.317	
" 30	"	0.386		" 3	"	0.319	
" 31	"	0.385	† 0.382	" 3	"	0.320	† 0.311
Sept. 23	M.H.V.	0.324		" 5	"	0.317	
" 23	"	0.337	† 0.316				

\* The first three samples analyzed were prepared by introducing the methane into a special combustion pipette from which it was added directly to a volume of outdoor air already in the burette. The remaining analyses were made on samples obtained from mixtures prepared in the Douglas bag as already described.

† Analyses within the brace were made from one sampling tube.

‡ This was the same air mixture as that analyzed on September 23.

Illustrative analysis of mixed sample of outdoor air and methane:

Computed methane in Douglas bag = 0.289 per cent.

	Burette reading.	Correction.	Corrected reading.
Initial reading.....	99.950	+ 0.108	= 100.058
After absorption of CO <sub>2</sub> in sample.....	99.920	+ 0.108	= 100.028
Contraction.....			0.030

$$\text{Carbon dioxide} = \frac{0.030}{100.058} = 0.030 \text{ per cent.}$$

## Combustion of methane.

Gas passed 10 times over wire.....	99.425		
CO <sub>2</sub> absorbed.....	99.190		
Gas passed 10 times over wire.....	99.140		
CO <sub>2</sub> absorbed.....	99.115		
Gas passed 5 times over wire.....	99.090		
CO <sub>2</sub> absorbed.....	99.090	+	0.110 = 99.200
Total contraction.....			0.828

$$\text{Methane found} = \frac{0.828}{3} = 0.276 \frac{0.276}{100.058} = 0.276 \text{ per cent.}$$

The determinations made on different mixtures of methane and outdoor air are given in Table I, in which are shown duplicate determinations on the same gas compared with the computed percentages of methane presumed to be in the mixtures.

One of the difficulties encountered in this work was that of making sure that the Douglas bag was completely empty before introducing gas. This is not easily done and consequently some of the values may be lower than the theoretical percentages calculated for the mixtures because of the presence of some air in the bag before the methane was admitted.

The apparatus is now in constant use at the Institute of Animal Nutrition of the University of New Hampshire.



## THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL.

### II. A SEPARATION INTO AN ACTIVE AND AN INACTIVE FRACTION.

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(Received for publication, June 29, 1926.)

\* As is well known, young rats can be regularly protected from rickets by the addition to their dietary of 1 mg. or less of irradiated cholesterol. Various experiences led us to believe that this cholesterol was a mixture of active and inactive material and that an attempt should, therefore, be made to fractionate it. In the first place prolonged irradiation gradually inactivates cholesterol which has been rendered antirachitic by means of ultra-violet rays. According to our conception of the process, cholesterol in the course of irradiation is being inactivated at the same time that it is being activated, and thus represents a mixture of at least two forms of cholesterol. Another experience which induced us to undertake fractionization experiments was the recent report of Beumer (1) to the effect that activated cholesterol could be separated into a digitonin-precipitable and non-precipitable substance. His tests did not include an investigation as to whether the non-precipitable residue of irradiated cholesterol possessed antirachitic potency. As reported in a recent communication (2), an attempt was made to obtain an active fraction of irradiated cholesterol by means of the well known method of precipitation with an alcoholic solution of digitonin. The filtrate was evaporated to dryness *in vacuo*, the white residue taken up and washed with small volumes of ether, and the solution then evaporated *in vacuo*. This amorphous residue, as well as the digitonin precipitate, was fed to series of rats, but much to our surprise neither

fraction showed any protective power. Several experiments of this kind were carried out without success.

Recently we have modified the technique of fractionization in several particulars; the separation has been carried out in an atmosphere of nitrogen, the oily menstruum in which the fractions are suspended was mixed immediately with the fractions, and curative rather than prophylactic tests were employed in order to shorten the period of the tests. This curtailment was resorted to with the idea that our failure to obtain protection might have resulted from a rapid deterioration of the fraction. The method was as follows:

The separation of the cholesterol digitonide from the soluble fraction was carried out in an atmosphere of nitrogen, using a modification of Steinberg's apparatus for suction filtration. After thorough washing of the precipitate, linseed oil was run directly into the receiving flask and the alcohol distilled off *in vacuo*. Previous experiments show that the active fraction so obtained is about 4.5 per cent of the total irradiated cholesterol.

The precipitated digitonide was mixed with linseed oil on the filter plate before transferring to a flask. Because of the very limited solubility of this fraction in the oil, it is obvious that exposure to oxygen was not avoided.

When series of rats were fed the digitonin-precipitable and the non-precipitable fractions prepared in this way, a clear cut result was obtained; those animals receiving the former fraction showed no subsequent healing of the rachitic process, whereas those which received the non-precipitable fraction gave evidence of marked healing within the experimental period of 9 days. Table I shows that the percentage of inorganic phosphate of the blood was, as would be expected, definitely higher in the latter group. The active fraction constituted only about 4 to 5 per cent of the original amount of cholesterol which had been irradiated for a period of  $\frac{1}{2}$  hour at a distance of 1 foot.

This experiment is of interest as providing a method of concentrating activated cholesterol, as well as indicating that only a small part (approximately 5 per cent) of activated cholesterol possesses antirachitic properties. Its greater interest, at the present time, would seem to lie in the fact that the result links the specific antirachitic power of activated cholesterol with that

TABLE I.  
*Antirachitic Activity Digitonin-Precipitable and Non-Precipitable Fraction of Irradiated Cholesterol.*

Rat No.	Weights. <i>gm.</i>	Rickets-producing diet.	Rickets previous to test.	Supplement to diet.	Subsequent healing (9 days).			Blood P (inorganic).
					Radiographic.	Macroscopic (silver stain).	Microscopic.	
10618	30-50	Low phosphorus No. 84.	Moderate.	2.5 mg. digitonin-precipitable fraction.	No. " " "	No. Very slight. No. "		no.
10620	34-40		"					
10621	35-44		"					
10622	30-40		"					
10623	30-40	Low phosphorus No. 84.	Moderate.	2.5 mg. digitonin-non-precipitable fraction.	Marked. " " "	Marked. " " "		3.07
10624	30-44		"					
10625	34-40		"					
10639	28-30		"					

of cod liver oil. As is well known, the antirachitic potency of cod liver oil has been found to be due entirely to its non-saponifiable fraction. Furthermore, it has been shown by Dubin and Funk (3) that this fraction can be rendered still more potent by means of "eliminating the cholesterol" by precipitation with digitonin. Coupling these fractionization experiments of cod liver oil and of activated cholesterol, we have good evidence to the effect that a close chemical similarity exists between the active principle of these two substances, and that their protective and curative action in rickets is due to a factor common to both. Probably the activity of cod liver oil is to be ultimately ascribed to ultra-violet radiation either directly of the cod itself, or more probably, indirectly through the food.

In a short communication Koch, Cahan, and Gustavson (4) have reported recently an experiment in which, "The non-saponifiable fraction of cod liver oil was extracted with liquid ammonia and again a brown, gummy residue was obtained. This fed in daily doses of 2 mg. prevented rickets entirely." In view of this result we carried out a series of experiments to ascertain whether activated cholesterol could be fractionated similarly by means of liquid ammonia. The process was as follows:

Anhydrous liquid ammonia was run into a reaction cylinder containing irradiated cholesterol and allowed to remain in contact with the substance, under pressure from the ammonia tank, for 3 hours. After that period the ammonia solution was forced through an asbestos filter and run slowly into a large volume of distilled water. The entire system was flushed thoroughly with ammonia from the tank. The water containing the ammonia-soluble fraction was evaporated nearly to dryness. Linseed oil was then added and the water driven off completely. About 96 per cent of the original amount of irradiated cholesterol was recovered from the reaction chamber. This confirms an ammonia-soluble fraction of somewhat less than 4 per cent, as indicated by direct weights in preliminary experiments. Quantitative determinations were not made.

Rats which were given this preparation in daily amounts of 2.5 mg. were protected from rickets while fed on the standard low phosphorus diet. This result furnishes once more an analogy

between the active antirachitic substance in cod liver oil and that in cholesterol which has been treated with ultra-violet radiations.

Liquid ammonia has been employed successfully by these investigators in obtaining an active fraction, the female sex or ovarian hormone, from follicular fluid and other tissue. This hormone bears certain resemblances to activated cholesterol; both are lipoids of high molecular weight, both unsaturated compounds, and show a high degree of thermostability. In view of these points of similarity, it was thought worth while to investigate whether the female sex hormone possessed any antirachitic properties and also whether irradiated cholesterol showed any of the specific activity of the sex hormone. The fraction obtained from activated cholesterol by means of liquid ammonia was tested by Dr. Robert Frank, but failed to bring about contractions of the uterus when injected subcutaneously in doses of 2.5 mg. This amount of the extract of ovarian follicular fluid suffices to bring about this characteristic reaction. Furthermore, the fraction containing the ovarian hormone, which had been extracted from follicular fluid, shows no antirachitic properties when tested on rats. It is evident, therefore, that in spite of various chemical points of similarity, these two factors are quite different in their essential properties.

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## THE LIBERATION OF ADSORBED SUBSTANCES FROM THE PROTEINS.

### II. THE EFFECT OF ADDITION OF SODIUM OLEATE TO WHOLE BLOOD UPON THE NON-PROTEIN NITROGEN IN BLOOD FILTRATES.

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(Received for publication, July 8, 1926.)

We have previously shown (1) that certain dye stuffs which attach themselves to the proteins may to varying degrees be freed from such a union by the action of substances which lower surface tension. Especially marked is the effect of bile salts and sodium oleate; this is largely due to their great affinity for proteins, whereby they associate themselves with the protein molecules and tend to displace other substances which are less strongly attracted to the proteins than themselves. In the previous experiments ultrafiltration through collodion membranes was employed; it was demonstrated that dye stuffs which were diffusible in aqueous solution would be held back from the ultrafiltrate if proteins were added to the solution, and that the dye would reappear in the ultrafiltrate when bile salts were added to the solution of dye and protein in the ultra filter.

A similar action may be shown by precipitation of the proteins and estimation of the dye in the filtrate. Marshall and Vickers (2) have found by ultrafiltration that phenolsulfonephthalein, when added to dog blood in not excessive concentration, will be bound to the extent of 55 to 65 per cent. This may be simply demonstrated by adding 0.3 mg. of phenol red to 1 cc. of blood and precipitating the proteins with tungstic acid according to the technique of Folin and Wu (3). The filtrate will contain approximately 45 per cent of the dye. If sodium oleate be added to the blood before precipitation of the proteins, the amount of dye

which appears in the filtrate will be increased considerably. With 25 mg. of oleate per cc. of blood, 62 per cent of the dye can be recovered from the filtrate (see Table I). This represents an increase of 37 per cent of the amount of dye originally in the filtrate.

Bromsulphalein, a dye which is rapidly excreted in the bile but which appears in the urine only in traces, binds itself firmly to the proteins. Excessive quantities must be added to the blood before

TABLE I.

The effect of addition of sodium oleate to whole blood upon the amount of phenol red recovered in the protein-free blood filtrate. 0.3 mg. of phenol red added to each cc. of whole blood. Tungstic acid used as precipitant.

	Mg. of oleate per cc. of blood.			
	0	6.25	12.5	25
Per cent of phenol red in blood filtrate.	43.5 46.5	45.5 47	53.2 53	62.5 62

TABLE II.

The effect of sodium oleate upon the amount of bromsulphalein recovered in protein-free blood filtrates. 10 mg. of bromsulphalein and 0 to 25 mg. of sodium oleate added to each cc. of whole blood. Tungstic acid used as precipitant.

	Mg. of oleate per cc. of blood.				
	0	3.12	6.25	12.5	25
Per cent of bromsulphalein in blood filtrate.	0.45 0.46 0.52	0.68	0.72 0.70	0.88 0.98 1.18	2.06 1.57 1.64

appreciable amounts appear in the filtrates. Upon the addition of 10 mg. of bromsulphalein to 1 cc. of dog blood, the protein-free blood filtrate will be found to contain only 0.45 to 0.52 per cent of the quantity added. However, with blood containing 25 mg. of sodium oleate per cc. from 1.57 to 2.06 per cent can be filtered off. This is an average increase of over 350 per cent of the dye in the filtrate. These results are of interest in consideration with the findings reported below (Table II).

Upon applying this principle to the non-protein nitrogen of the blood, it was found that considerable increases could be brought about by the addition of sodium oleate to whole blood before precipitation of the proteins. Sodium oleate was employed because a nitrogen-free product of this compound could be obtained. The proteins were precipitated with 10 per cent sodium tungstate and  $\frac{2}{3}$  normal sulfuric acid according to the technique of Folin and Wu. From 5 to 25 mg. of sodium oleate in 0.5 per cent aqueous solution and water to make 7 cc. of solution were added to each cc. of whole blood. Quantities of oleate larger than 25 mg. per cc. of blood could not be used because of the difficulty of obtaining protein-free filtrates. The oleate solution was slightly alkaline, but this alkalinity was estimated and compensated for by the addition of an equivalent quantity of sulfuric acid in excess of that required for precipitation. To exclude the possibility that the alkalization caused an increase of the non-protein nitrogen through hydrolysis of the proteins, 0.4 cc. of 0.1 normal sodium hydroxide (corresponding to the alkalinity of 25 mg. of oleate) was added to each cc. of dog blood. Estimations of the non-protein nitrogen showed no increase over that present in control samples.

The filtrates employed were all water-clear and gave negative tests for protein with heat and acetic acid, nitric acid ring test, sulfosalicylic acid, as well as the rose bengal test which will detect the presence of proteins in dilutions of 1 to 4 millions (4). The sodium oleate, in the quantities added, became completely bound to the proteins so that practically none appeared in the blood filtrates.

Total non-protein nitrogen determinations were carried out with the micro-Kjeldahl method of Folin and Wu, employing 5 cc. of blood filtrate. After digestion with the acid mixture, the nitrogen was determined either by direct Nesslerization, or by distillation and estimation of ammonia in the distillate by Nesslerization or by titration. When the distillate was Nesslerized, it was collected in 0.1 normal hydrochloric acid, while 0.01 N acid and alkali were used when titration was employed. Estimations were simultaneously carried out upon two separate control samples of blood, and upon three samples to which 6.25, 12.5, and 25 mg. of oleate had been added.

*Results.*

Seven series of determinations were made upon blood from four dogs. In every instance considerable increases in the non-protein nitrogen in the blood filtrates were brought about. These amounted to 8 to 20 mg. above the normal values when 25 mg. of oleate were added to each cc. of whole blood. Such increases represented 20 to 55 per cent, with an average of 35 per cent more than the non-protein nitrogen present in the control samples to which no oleate had been added.

The increase in non-protein nitrogen brought about by the addition of 12.5 mg. averaged 23 per cent above normal. This is

TABLE III.

The non-protein nitrogen in blood filtrates obtained from blood to which varying amounts of sodium oleate had been added before precipitation of the proteins. Tungstic acid used as precipitant.

	Dog No.	Mg. of oleate per cc. of blood.				
		0	0	6.25	12.5	25
Mg. per cent of non-protein N in blood filtrates.	1	36		42	49	56
	1	36		40	51	
	2	33.2	35			48
	3	32		36	38	41.2
	4	35				46
	4	37				46.8
	4	37	37.4	39	43	49.5

somewhat more than half of the increase caused by 25 mg. of oleate, and such a relation was anticipated to occur if we were dealing with an adsorption process—that is, decreasing quantities of oleate should be relatively more effective in the liberation of adsorbed substances. The increases, however, with 6 mg. of oleate per cc. of blood were irregular; an average increase of 10.5 per cent occurred (Table III).

These findings are of considerable theoretical interest and their significance will be more fully understood when the nature of the substances which are liberated from the proteins is known. Urea, because of its great diffusibility, is probably not at all bound to the proteins. Amino acids (5) have been recovered quantitatively when added to the blood, and this would indicate that no portion of them is held back by the proteins, although conditions may not be comparable because an excess of these substances is added in

such experiments. It seems likely that the "rest nitrogen" of the blood may be chiefly involved in the increases we have brought about. The rest nitrogen comprises approximately 46 per cent of the total non-protein nitrogen and the nature of the substances which compose it is at present unknown.

It is worth pointing out that Hiller and Van Slyke (5) using seven different protein precipitants obtained no appreciable variation in the total non-protein nitrogen except with ethyl alcohol, which gave a decrease due to the fact that amino acids were held back by the proteins when this precipitant was used.

The question arises—what is the actual value for non-protein nitrogen in normal blood? It was shown that with phenol red, which is bound to the proteins of dog blood slightly over 50 per cent, sodium oleate caused an increased concentration in the blood filtrate amounting to 37 per cent. On the other hand, with bromsulphalein, which is almost completely bound to the proteins, similar quantities of oleate increased the concentration of the dye in the filtrate several hundred per cent. The results obtained with non-protein nitrogen are comparable to those found with phenol red, and this suggests that approximately half of the non-protein nitrogen is thrown down with the proteins in the usual methods of obtaining protein-free filtrates for blood analysis.

Besides the intrinsic interest of these experiments, they represent a line of investigation which can be applied to numerous other problems concerned with the chemical analysis of proteins and protein-containing fluids.

#### SUMMARY.

By the addition of 25 mg. of sodium oleate per cc. of whole blood, it is possible to increase the non-protein nitrogen in the blood filtrates from 20 to 55 per cent. This increase is due to the liberation of non-protein nitrogenous substances which ordinarily remain attached to the proteins and do not appear in the filtrates.

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## THE PHYSIOLOGICAL SIGNIFICANCE OF DEAMINATION IN RELATION TO GLUCOSE OXIDATION.

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In both plant and animal tissues the deamination of amino acids is of wide spread occurrence and a variety of products are formed. The exact distribution of the process and its chemical mechanism in the animal body are still subjects of importance from the physiological standpoint, but perhaps of greater importance is the question of the influence of amino acid decomposition on the total metabolism of the cell. The phenomenon of the "specific dynamic action" of protein and amino acids has been established by several workers, and the literature has been fully reviewed by Lusk (1). Although the facts have been established experimentally the mechanism by which total metabolism is stimulated by deamination still remains a matter for hypothesis. In connection with our work on the metabolism of bacteria experimental results have been obtained which support the view that this stimulation is primarily due to the  $\text{NH}_3$  liberated by deamination. Later in this report we shall consider more fully the possible bearing of our work on the problem of carbohydrate oxidation in the animal body.

The organism used in this investigation was the typical form, *Bacillus granulobacter pectinovorum*, which produces acetone and butyl alcohol in media containing utilizable carbohydrates. It is a bacillus which hydrolyses starch to glucose; which is in turn oxidised anaerobically. In previous reports we have shown that butyric acid and acetic acid are intermediates in this process of oxidation (2). Peterson, Fred, and Domogalla have demonstrated experimentally that during the fermentation the protein present in the medium is hydrolysed as far as the amino acid stage (3), and by the isolation of *L*-leucic acid have indicated the pos-



sibility of a general deamination of the liberated amino acids (4). Our experiments were performed with the primary object of correlating more closely (a) vegetative growth of the cells, (b) oxidation of glucose and intermediate fatty acids, and (c) deamination of amino acids and the accumulation or utilization of the products.

*Relationship between Growth and Oxidation.*

*Experiment I.*—A flask containing 1000 cc. of 5 per cent maize mash was sterilized for 2 hours at 15 pounds steam pressure. The medium was cooled and inoculated with 20 cc. of an active culture of the bacillus in the same type of medium. The flask was incubated at 37°C., and at regular intervals samples of the

TABLE I.

Time after inoculation.	0.1 N acid in 10 cc. of mash.	Vegetative growth.	Morphological observations.
hrs.	cc.	per cent	
0	0.45	3.5	Scattered rods.
2.0	0.60	6.0	
4.0	0.80	11.0	Long filaments.
6.0	1.30	60.0	Chains, rods evenly stained.
10.5	3.20	100.0	Rods, a few clostridia.
14.5	4.35		Clostridia, granular rods.
24.5	1.70		
29.5	1.80		
48.0	2.30		

fermenting mash were taken under aseptic conditions. Portions of each sample were titrated with 0.1 N NaOH to obtain the acidity curve of the fermentation. A standard loopful of culture was spread evenly over 2 sq. cm. on a slide, carefully fixed by heat, and stained with methylene blue. Five fields in each preparation were counted, and at the same time observations were made regarding the general morphological condition of the culture. By combining the direct counts and these observations we consider it possible to determine accurately the limits of the period of vegetative growth. The results from this experiment are summarized in Table I. The counts are expressed as percentages of the highest figure.

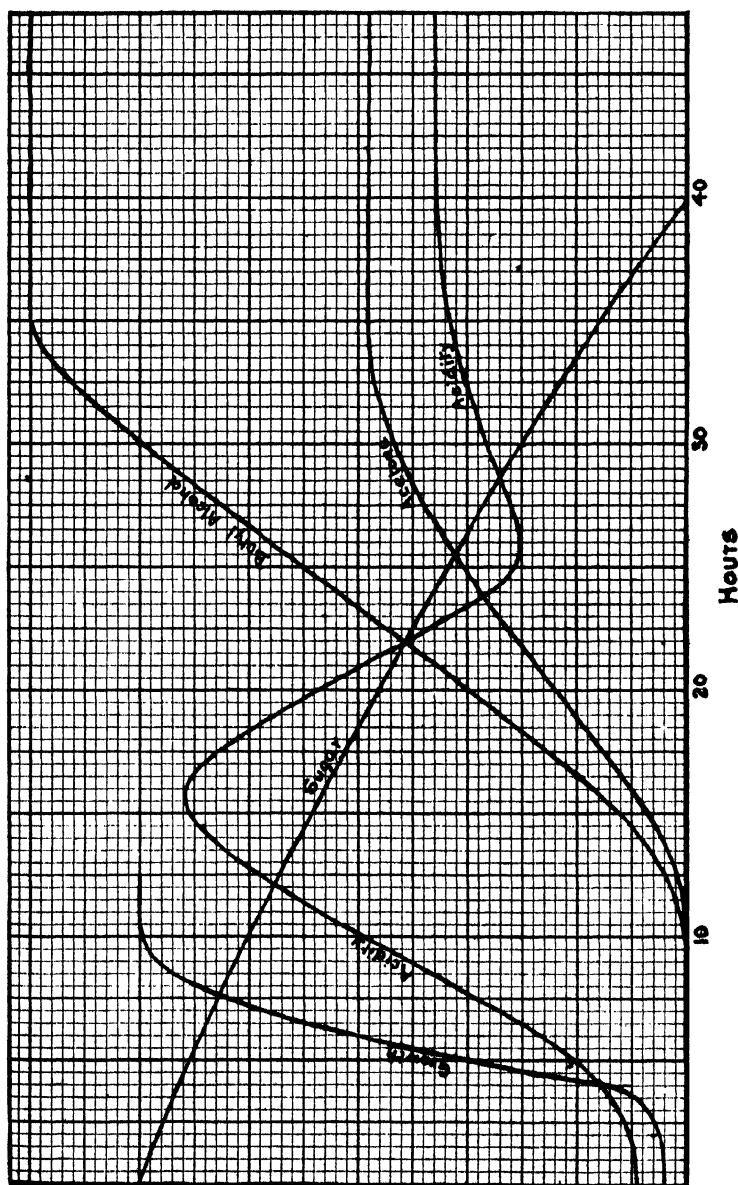


CHART 1. A diagrammatic representation of a normal fermentation showing the relationship in time between sugar utilization, vegetative growth, and the accumulation of primary and secondary products of oxidation.

After about the 12th hour of the fermentation period the starch had become sufficiently hydrolysed to pass into solution, and the medium was slimy. The organisms began to lose their motility and congregated in dense groups, a condition which made bacterial counts by any method difficult and inaccurate. That vegetative growth had ceased was made quite clear, however, by comparing slides made at this stage with earlier ones showing the culture in a condition of vigorous cell division. Furthermore, the appearance of the characteristic spindle-shaped cells or clostridia containing miniature endospores is an indication of a cessation of cell division in a normal fermentation. This point was reached about 4 hours previous to the peak of the acidity curve.

In Chart 1 the results obtained in this experiment have been correlated with previously established facts regarding glucose oxidation (2). The curves in Chart 1 demonstrate very clearly the existence of two distinct phases of the fermentation period. During the first 10 to 12 hours the cells added to the medium divide rapidly and the acidity rises. At the same time roughly 30 per cent of the carbohydrate is utilized. During the remainder of the fermentation the cells no longer multiply but follow one of two paths, changes leading to spore formation or slow autolysis and disintegration. These morphological changes are accompanied by (a) a rapid primary oxidation of glucose, (b) a marked increase in the rate of oxidation of intermediate fatty acids leading to a fall in the free acidity of the medium, and (c) the formation of characteristic neutral end-products, butyl alcohol and acetone. The true fermentation period is that during which the cells are passing into the resting state or breaking up. Grey has shown that during a period of cell disintegration *Bacillus coli communis* oxidises glucose with great rapidity (5).

#### *Process of Deamination.*

In addition to the volatile acids which are formed from glucose an acid fraction accumulates, particularly during the second phase of the fermentation, which is non-volatile. From this fraction Schmidt, Peterson, and Fred (4) isolated *l*-leucic acid and prepared its zinc salt which they submitted to a combustion analysis. They concluded that it was derived from *l*-leucine and

suggested that a more general deamination occurs during the fermentation. I propose to confirm very briefly these observations and to support their suggestion.

*Experiment II.*—100 liters of maize mash were sterilized and fermented through to completion. The material was filtered through muslin and evaporated down to a thick syrup under reduced pressure at a temperature of 50°C. This treatment removed volatile acids and neutral products. The syrup was divided into 250 cc. portions, which were acidified with  $\text{H}_2\text{SO}_4$ , and extracted continuously for 4 days with ether. Distilled  $\text{H}_2\text{O}$  was added to the ether in the receiver, and the ether was removed. The residue was an amber-coloured liquid containing acid products. This solution was made slightly alkaline with  $\text{Ba}(\text{OH})_2$ , and extracted again with ether to remove colouring matter. The mixture of barium salts in aqueous solution was boiled for

TABLE II.

Material.	Weight of sample.	Zn	C	H <sub>2</sub>
	gm.	per cent	per cent	per cent
Experimental (a).....	0.2045	20.25	42.81	6.412
“ (b).....	0.2689	20.43	42.70	6.493
Schmidt, Peterson, and Fred.....		19.93	44.15	6.66
Zn hydroxyisocaproate.....		19.96	43.97	6.60

2 hours with  $\text{ZnCO}_3$  under a reflux condenser. The solution of zinc salts was reduced in volume and allowed to crystallize. The largest portion obtained in a pure form by recrystallization from  $\text{H}_2\text{O}$  was composed of long rhombic crystals. Two portions of this material were submitted to ultimate analysis by my colleague Professor L. Rogers. His results are given in Table II. It was not considered necessary to repeat further the methods adopted by Schmidt, Peterson, and Fred in their identification of *l*-leucic acid.

In addition to *l*-leucic acid we obtained a small amount of an acid product which had the following chemical properties. Its barium salt was decomposed in aqueous solution by  $\text{CO}_2$ . With Millon's reagent a dilute solution formed a deep red colouration when the two were shaken together in the cold, indicating the presence of a phenolic ring. Koessler and Hanke (6) diazo rea-

gents plus a trace of a solution containing the acid gave a reddish brown colour, which developed to its maximum intensity in 10 minutes and remained constant for several days. This colour was not changed by the addition of hydroxylamine hydrochloride and NaOH, showing that the side chain of the benzene ring, if any, does not contain an amino group. Solutions of the acid decolourized bromine water and alkaline potassium permanganate solution. The free acid crystallized from water in radially arranged groups of needles. In view of the above chemical data and the isolation of *l*-leucic acid by independent observers we conclude that tyrosine is also deaminated during the fermentation, and that *p*-hydroxyphenyllactic acid is produced. Our residue of salts contained traces of at least two other acids which is further evidence of a general deamination of amino acids.

*Correlation of Deamination, Ammonia Production, and Oxidation.*

The non-volatile acids are formed almost entirely when vegetative growth has ceased; *i.e.*, during the period of most vigorous oxidation. If we could assume that the whole of this acid material is derived from protein it would be possible to conclude without further experimentation that deamination is associated in time with vigorous intracellular oxidation and not with vegetative growth. A part of the non-volatile acid material may, however, be derived from carbohydrate, but there is no possibility that *p*-hydroxyphenyllactic acid has this origin. We therefore followed the course of deamination by measuring the rate of production of this acid in the fermentating mash, and, at the same time we observed the rate and extent of ammonia formation.

*Experiment III.*—A flask containing 1500 cc. of 5 per cent maize mash was sterilized and inoculated with 20 cc. of an active culture. The titratable acidity curve of the fermentation was obtained in the usual way. At intervals ammonia determinations were made by the aeration method, using 50 cc. samples. The estimations of *p*-hydroxyphenyllactic acid were made in the following manner.

To 10 cc. of mash add 25 cc. of ether in a separating funnel and shake mechanically for 10 minutes. Remove the aqueous layer and add 5 cc. of 1.1 per cent  $\text{Na}_2\text{CO}_3$  to the ether. Shake for 10 minutes. Run the carbonate solution into a cup of the

colorimeter and add 2 cc. of the Koessler and Hanke diazo reagent with 1 cc. of distilled  $H_2O$ . Shake and allow to stand for 10 minutes. Compare with a standard of Congo red and methyl orange.

When the acidity of the fermenting mash was at its maximum a large sample was withdrawn and incubated with an excess of toluene in order to arrest any endoenzyme activity. From this point onwards readings were made on the fermenting and non-fermenting mash. The results from the experiment are given in Table III.

TABLE III.  
*Deamination and  $NH_3$  Production.*

Time after inoculation.	Normal fermentation.			Mash + toluene.	
	0.1 N acid in 10 cc.	p-hydroxy-phenyllactic acid.	0.1 N $NH_3$ in 50 cc.	0.1 N acid in 10 cc.	p-hydroxy-phenyllactic acid.
hrs.	cc.	mm.	cc.	cc.	mm.
13	4.0		0		
16	4.4	2.0			
20	4.0	3.0	0	4.0	0
25	3.5	23.0	0		
37	2.1	74.0			
43	2.3	75.0	0.1		
68	2.8		0.2	3.9	0
92				3.9	0

The experimental results lead to the following conclusions: Deamination is an endocellular process and it occurs mainly during the second phase of the fermentation period; *i.e.*, when the cells are passing into the spore form or disintegrating and the oxidation of glucose and intermediate fatty acids is most vigorous. During this period the hydroxy acids formed from the amino acids accumulate in the medium, but none of the liberated  $NH_3$  diffuses out from the cells. When the fermentation has practically ceased a trace of  $NH_3$  can be detected. At the end of a normal fermentation similar to that in Experiment III 50 cc. of mash contain about 5 cc. of 0.1 N acid which is non-volatile and only 0.2 of 0.1 N  $NH_3$ .

The question then arose as to whether the deamination process and carbohydrate oxidation are connected physiologically

in addition to running parallel in time. Assuming some connection to exist, it seemed logical to enquire more fully into the effect of  $\text{NH}_3$  utilization during the period of the fermentation when the cells are in the resting state.

*Ammonium Phosphate as a Catalyst of Oxidation.*

Before attempting to investigate the influence of  $\text{NH}_3$  on the oxidation of glucose it was necessary to determine whether  $\text{NH}_3$  was a suitable source of N for the organism during the period of protoplasmic synthesis and vegetative growth. We inoculated a large number of different media containing glucose, mineral salts, and different ammonium salts in sufficient concentration to equal the N present in maize mash. To each flask containing 200 cc. of medium we added 2 cc. of an active culture of the bacillus. We observed no signs of vegetative growth, and the media were not fermented. A similar amount of inoculum in media containing protein, peptone, or a mixture of amino acids gives rise to a vigorous and complete fermentation. We concluded from these experiments that  $\text{NH}_3$  in the form of salts will not support vegetative growth.

*Experiment IV.*—We next attempted by the use of larger volumes of inoculum, and therefore a larger number of active cells, to study the effects of ammonium phosphate on the oxidative processes of the cells. Six 300 cc. flasks containing 200 cc. of medium were sterilized in the usual manner. The basis of the medium was a mineral salt solution of the following composition.

$\text{K}_2\text{HPO}_4$ .....	0.5 gm.	$\text{FeSO}_4$ .....	0.01 gm.
$\text{KH}_2\text{PO}_4$ .....	0.5 "	$\text{NaCl}$ .....	0.01 "
$\text{MgSO}_4$ .....	0.2 "	$\text{C}_6\text{H}_{12}\text{O}_6$ .....	30.00 "
$\text{MnSO}_4$ .....	0.01 "	$\text{H}_2\text{O}$ .....	1000.00 cc.

Two flasks, A and B, contained the above medium, Flasks C and D the same with the phosphates doubled in amount, and Flasks E and F the same plus 0.5 gm. of  $\text{NH}_4 \cdot \text{H}_2\text{PO}_4$  and 0.5 gm. of  $(\text{NH}_4)_2\text{HPO}_4$  per liter. Each flask was inoculated with 10 cc. of an active culture of the bacillus in maize mash, and an Atwood valve containing  $\text{H}_2\text{SO}_4$  was inserted in each in place of the cotton plug. The flasks were weighed before and during the period of incuba-

tion. The losses in weight due to gas production are indications of the rate and extent of glucose utilization. The results from the experiment are given in Table IV and in Chart 2.

*Experiment V.*—Four Erlenmeyer flasks each containing 500 cc. of medium were prepared and sterilized. Two contained

TABLE IV.  
*Ammonium Phosphate and Oxidation.*

Flask.	Loss in weight after:							
	22 hrs.	44 hrs.	68 hrs.	96 hrs.	115 hrs.	140 hrs.	164 hrs.	188 hrs.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
A.....	0.17	0.43	0.67	0.86	0.96	1.02	1.09	1.16
B.....	0.16	0.36	0.56	0.68	0.72	0.78	0.82	0.84
C.....	0.18	0.41	0.63	0.80	0.88	0.98	1.08	1.15
D.....	0.18	0.39	0.61	0.77	0.86	0.94	1.02	1.10
E.....	0.17	0.32	0.52	0.89	1.19	1.52	1.80	2.08
F.....	0.17	0.31	0.48	0.77	1.09	1.41	1.65	1.80

TABLE V.

Time after inoculation.	Flask A.	Flask B.	Flask C.		Flask D.	
	Acid.	Acid.	Acid.	NH <sub>3</sub>	Acid.	NH <sub>3</sub>
hrs.	cc.	cc.	cc.	cc.	cc.	cc.
0	0.8	0.8	1.2	1.2	1.1	1.2
24	2.3	2.3	2.4		2.4	
48	3.4	3.3	3.4	1.2	3.6	1.2
76	3.4	3.4	3.7		3.7	
100	3.4	3.4	3.8	1.2	3.7	1.0
120	3.3	3.4	3.8		3.7	
144	3.3	3.3	3.7	1.2	4.2	0.8
168	3.2	3.3	3.7		3.7	
192	3.2	3.3	4.2	0.9	3.0	0.7
220	3.2	3.3	3.7		2.8	
240	3.1	3.4	3.2	0.75	3.0	0.5

mineral salts and glucose but no source of nitrogen, and the other pair contained a similar medium with the addition of ammonium phosphate equal to the concentration used in Experiment IV. The flasks were inoculated with 20 cc. of an active culture and incubated at 37°C. At regular intervals determinations of the total free acidity and ammonia were made. The residual sugar



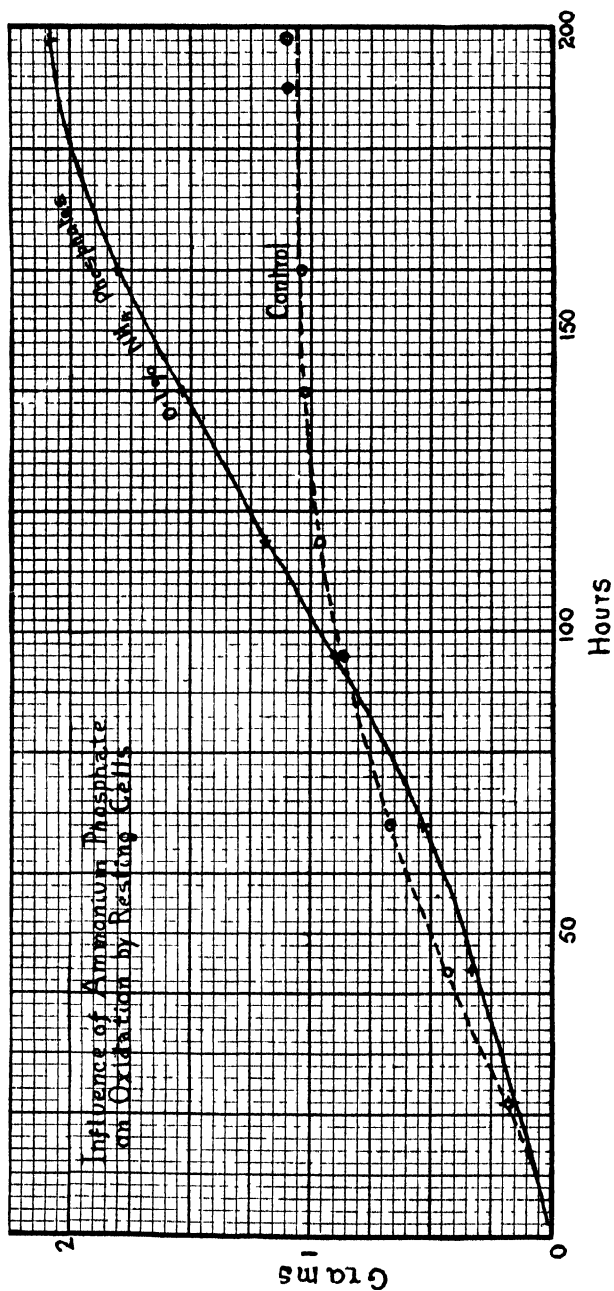


CHART 2. Curves showing the influence of small quantities of ammonium phosphate on the rate of fermentation.

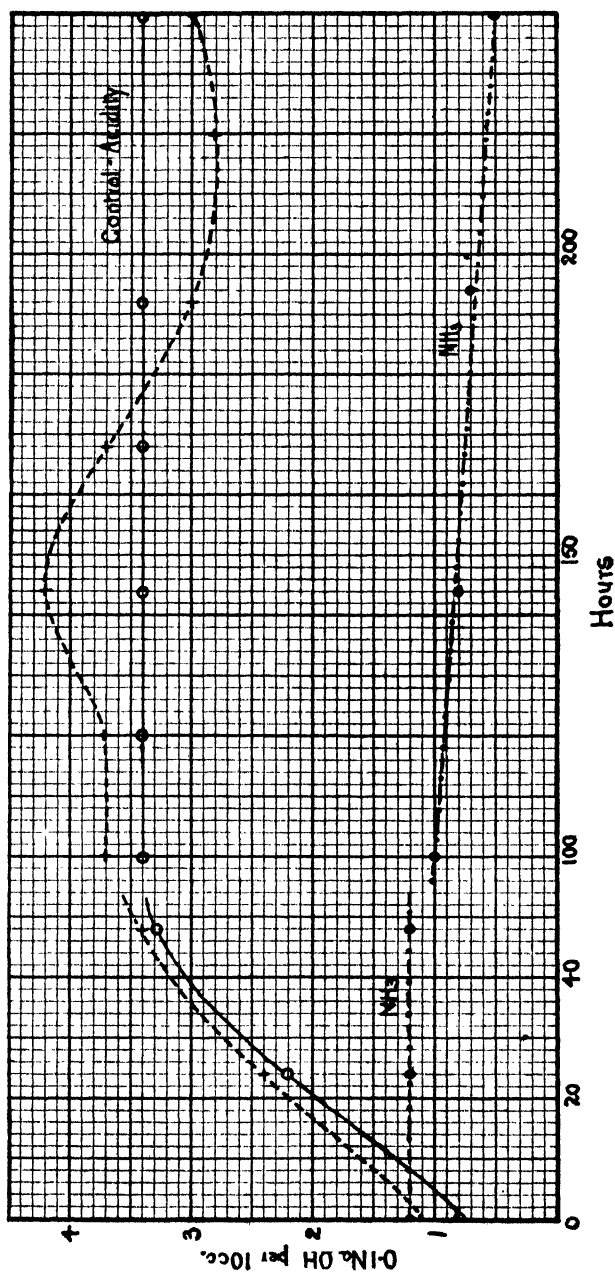


CHART 3. The curves are based on Experiment V and show the relationship between ammonia utilization and the normal periods of growth and fermentation.

in each flask was also determined. The results from this experiment are given in Table V and Chart 3.

During the first 3 days of the incubation period the fermentations were almost identical. The accumulation of free acid, *i.e.*, primary oxidation products, proceeded at the same rate in all cases, and at the end of this period the acidity curves were running horizontally. It is during this earlier part of the total fermentation period that we should expect vegetative reproduction to take place, and the results show that if cell division and synthesis took place at all they did not involve the utilization of measurable amounts of  $\text{NH}_3$  in Flasks C and D. On the 10th day the concentration of  $\text{NH}_3$  in Flask D began to fall, and this continued until the close of the experiment. Its effect on the general character of the fermentation was very striking. The medium until this point had remained transparent, and only occasional bubbles of gas could be seen rising from the strips of filter paper at the bottom of the flask, but in a short time the supernatant liquid became slimy and charged with gas, capped by a deep layer of foam. The acidity rose slightly and then fell to a level below that of the controls, an indication of increased primary and secondary oxidation. In Flask C the utilization of  $\text{NH}_3$  commenced 3 days later than in Flask D, and the period of vigorous fermentation was correspondingly delayed. This fermentation was still in progress when our last observations were made. The results regarding glucose utilization confirm our results in Experiment IV in which the extent of oxidation was measured by gas production. Both experiments have been repeated several times, and we consider that they supply convincing evidence of a stimulation of intracellular oxidation by a simultaneous utilization of free  $\text{NH}_3$ .

#### *Effect of Tyrosine on Oxidation.*

We have observed that the bacillus used in our experiments is unable to grow and multiply from a few cells in a medium containing a single amino acid as the sole source of N. We repeated Experiment V but substituted for the ammonium phosphate various amounts of pure tyrosine.

*Experiment VI.*—Four 300 cc. Erlenmeyer flasks each containing 200 cc. of medium were prepared and sterilized. Each

flask contained the usual salts, 6 gm. of glucose, and strips of filter paper. Two flasks contained no source of N, and two contained 0.2 gm. and 0.1 gm. respectively of tyrosine. After inoculation with 10 cc. of an active culture the flasks were equipped with Atwood traps containing concentrated  $H_2SO_4$ , and they were then incubated. During the fermentation period the losses in weight due to gas production were determined. When gas production had ceased in all the flasks a 10 cc. sample from each was used to determine the concentration of *p*-hydroxyphenyllactic acid. The colour value of the controls was 7.8 and 7.4 mm., and of the flasks containing tyrosine 30.6 and 40.8 mm. respectively. The losses in weight due to gas production are given in Table VI.

TABLE VI.  
*Influence of Tyrosine on Rate of Oxidation.*

Flask.	Loss in weight after:							
	22 hrs.	46 hrs.	70 hrs.	114 hrs.	140 hrs.	168 hrs.	216 hrs.	288 hrs.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Control.....	0.27	0.35	0.52	0.70	0.74	0.78	0.95	1.16
0.1 gm. tyrosine..	0.35	0.68	1.01	1.22	1.42	1.64	2.00	2.20
0.2 " " ..	0.31	0.62	0.92	1.11	1.32	1.53	1.83	2.01

The results from this experiment confirm our previous observation that the bacillus is able to deaminate tyrosine, and also that as a result the oxidation of glucose by the cells is catalyzed.

#### DISCUSSION.

It is unnecessary to discuss further the evidence for the existence in these anaerobic bacilli of a biochemical mechanism for the deamination of amino acids and the production of  $NH_3$  and hydroxy acids. The question which merits more detailed consideration is the physiological function of this process in the general metabolism of the cells. Koessler and Hanke (7) in their study of the decomposition of histidine by bacteria suggest three possible functions for deamination, and these we shall consider in the light of our own experimental findings.

1. Nitrogen is removed from an amino acid owing to its absence from the medium in more easily utilizable forms, *e.g.* am-

monium salts or nitrates, in order to support the vegetative growth of the organism. Such is the case when *Bacillus coli communis* grows in a medium containing carbohydrate and a single amino acid (8). The decomposition of the amino acid ceases when the  $\text{NH}_3$  has been liberated. This explanation is rendered untenable in connection with our results by the following facts. The organism does not grow and multiply when the N supply consists of ammonium salts or a single amino acid. In maize mash the organism grows rapidly, but deamination is chiefly, if not entirely, active when growth has ceased.

2. The acid produced by deamination is utilized as a source of C during synthesis or in respiration owing to the absence of carbon compounds such as glucose or glycerol. An example of this type of deamination is to be found in Raistrick's work on the utilization of histidine by various bacterial species in solutions containing only mineral salts and the amino acid (9). In our experiments there was in the medium an abundance of readily utilizable carbohydrate, and there is no evidence that the bacteria utilize the hydroxy acids formed by deamination.

3. Deamination may be resorted to by the cells as a method whereby the hydrogen ion concentration of the medium can be controlled. The acid products of glucose oxidation are neutralized by the liberated  $\text{NH}_3$ . This involves the decomposition of the acid products of deamination, otherwise the  $\text{NH}_3$  would have little effect. This cannot be the physiological explanation of our results. The product of deamination which is utilized by the cells is the  $\text{NH}_3$  and not the acid.

I propose, therefore, to ascribe to bacterial deamination an additional possible physiological function. During the anaerobic respiration of carbohydrates and fatty acids the rate of oxidation is stimulated, directly or indirectly, by a simultaneous deamination of amino acids within the cell. This effect is directly associated with the utilization of the liberated  $\text{NH}_3$ , and the hydroxy acids are secreted into the surrounding medium. The cycle through which the  $\text{NH}_3$  passes, and the precise mechanism by which its effect on oxidation is brought about are unknown. At the present time I know of no direct evidence from biological experiments which supports my conclusion, but some support is afforded by the *in vitro* experiments of Dakin (10) and Witzemann

(11) on the oxidation of butyric acid by means of  $\text{H}_2\text{O}_2$ . They found that the process of oxidation is catalyzed by the hydroxides of Na, K, and  $\text{NH}_3$ . Witzemann (12) in a later paper pointed out that the most marked effect is obtained by  $\text{NH}_4\text{OH}$ , and that it is not directly attributable to changes in the reaction of the substrate. He suggested that  $\text{NH}_4\text{OH}$  functions as a catalyst by giving rise to some unstable peroxide, but he also states that this peroxide is not formed from ammonium phosphates. On the basis of these experiments he advanced the hypothesis that  $\text{NH}_3$  acts as a catalyst of oxidation in the animal body, and called attention to the association in the liver of constant supplies of  $\text{NH}_3$  and a peculiar facility for the oxidation of such compounds as acetoacetic acid. Ray (13) has recently shown that the oxidation of lactic acid by  $\text{H}_2\text{O}_2$  is catalyzed by the presence of glycine, and he attributes this effect to the amino group.

Allowing for numerous differences there still remain many points of resemblance between the bacterial cells used in our experiments and the tissue cells of the animal body, and the question arises as to whether our results throw any new light on the mechanism of carbohydrate utilization in such cells. The phenomena relating to the "specific dynamic action" of protein and of single amino acids are sufficiently established, but the mechanism by which the total metabolism of the cells is stimulated remains a matter for hypothesis. Lusk (14) has suggested that the stimulus is due primarily to the hydroxy acids formed during deamination. Glycollic acid or lactic acid have not, however, the same quantitative influence as glycine or alanine in equivalent concentrations, and Lusk is led to postulate that "it may be that if deamination takes place within them with the production of glycollic acid, this substance may then play quite a different rôle from that which it plays when brought to the cell from without." If our results have any bearing on this aspect of the problem they provide a more definite answer to such questions. According to our view deamination takes place in the tissue cells, and hydroxy acids and  $\text{NH}_3$  are formed. The acids may or may not be further oxidised; if so, then they have a definite glucose value, but exert no specific dynamic effect. The  $\text{NH}_3$  in our experiments passed through some unknown cycle, but in the animal body the end of

the corresponding cycle is known, namely urea. According to this view we should expect to find the cause of the action of amino acids on total metabolism in the ammonia-urea cycle.

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# THE IDENTIFICATION OF ACETALDEHYDE IN NORMAL BLOOD AND ITS QUANTITATIVE STUDY IN THE BLOOD OF NORMAL AND DIABETIC DOGS.

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Evidence assigning to acetic aldehyde an important place in metabolic processes has accumulated steadily during recent years. Its function as an intermediate stage in the fermentative breakdown of carbohydrate has been reviewed by Neuberg (1). The theory that acetaldehyde plays a similar and equally important rôle in the oxidation of carbohydrate in the cells of the animal organism, is outlined by Gottschalk (2). There is also experimental foundation for the hypothesis that this labile substance is not only active in carbohydrate degradation and resynthesis, but that in addition it serves as a medium of exchange between fat, protein, and carbohydrate. Its significance from this point of view may be judged from summaries of late research on the intermediate stages of metabolism such as that of Nord (3).

The presence of traces of acetaldehyde in the animal body has been demonstrated, and it is known also that it can be formed in appreciable amounts by surviving tissues. Attention is directed to the report of Fabre (4) who found that acetaldehyde occurs in the blood of man and of the ox, dog, and horse in amounts varying from 0.2 to 0.6 mg. per liter, and that the aldehyde content may rise to 5 mg. per liter following pancreatectomy. These observations have been repeated and extended by Supniewski (5).

The finding of these two workers is directly in support of the contention that acetaldehyde has a central position in metabolism in the animal body. In view of the fundamental importance of their conclusions, the present authors undertook (1) to verify the statement that acetaldehyde is actually present in blood, and (2) to examine the methods on which Fabre and Supniewski base their claim that the aldehyde content of blood rises to several times its initial value, following the removal of the pancreas.



*I. Qualitative. Presence of Acetaldehyde in Ox Blood.*

As many liters of blood are necessary to furnish sufficient acetaldehyde to permit positive identification, the qualitative aspect of the investigation was confined to the blood of the ox.

*Principle of Method.*—The reagent selected for the isolation of acetaldehyde was 5,5-dimethylcyclo-hexandion-1,3 (dimethyl-hydroresorcinol), also known as dimedon, which was utilized by Neuberg in his "fixation" technique for binding the acetaldehyde produced during the course of fermentation. This material, first studied by Vorländer (6, 7), combines with aldehydes in the proportion of 2 molecules of dimedon to 1 of aldehyde, the condensation being accompanied by the elimination of 1 molecule of water. The procedure has been used by Stepp and Feulgen (8) in the identification of acetaldehyde in the urine of diabetics. The dimedon itself is relatively soluble in water, whereas its condensation product with acetaldehyde is only sparingly soluble and can be separated and identified by a melting point determination. Dimedon is an especially convenient reagent for the present work because it reacts with neither ketones nor sugars; in addition, aldomedon, its product of condensation with acetaldehyde, is approximately seven times the weight of the combining acetaldehyde, and thus it permits manipulation of small quantities.

The dimedon method was used by both Fabre (4) and Supniewski (5) for establishing the identity of the blood aldehyde, but unfortunately both authors report only in brief outline their results on this important phase of the problem. Their data will be referred to later in connection with the quantitative aspect of the present work.

*Clarification.*—After some experimenting, the tungstic acid method of Folin and Wu (9) in its original form was adopted for the removal of proteins from the blood. The centrifuge was used at first to remove the tungstic acid precipitate, but it was found that the process was slow and subsequent filtration of the supernatant liquid was required. Centrifuging was abandoned in favor of direct filtration. Large Whatman filter papers of No. 1 grade proved sufficiently rapid for this purpose and furnished a water-clear filtrate.

*Concentration.*—The distillation technique utilized by Stepp

and Feulgen (10) for concentrating normal urine for the identification of acetaldehyde was adopted here. Stepp and Feulgen were, however, interested primarily in a qualitative result, and before making use of the operation it was decided to apply it to a dummy solution of known low concentration in order to discover what losses may be expected.

At this stage of the work, acetaldehyde was estimated by the hydroxylamine method, which was first used for formaldehyde by Brochet and Cambier (11) and later modified by Sieber (12) for estimating acetaldehyde. According to Sieber's directions, the solution containing acetaldehyde is added to an excess of hydroxylamine hydrochloride. Acetaldoxime is formed after standing, and titration of the HCl liberated gives the equivalent acetaldehyde present. Hydroxylamine sulfate gave a sharper end-point, as recommended by Neuberg and Gottschalk (13).

To test the recovery of acetaldehyde by distillation, 20 cc. of a solution of acetaldehyde 0.0277 per cent by the hydroxylamine method were diluted to 8000 cc. in ice-cold water. A 2 liter distilling flask, a straight tube condenser, and a narrow receiver, conveniently a 100 cc. graduated cylinder, composed each still. The condenser tube dipped into 15 cc. of water at the bottom of the receiver, which was packed in ice at the commencement of operations and kept cold throughout. The 8000 cc. of solution were distilled in portions of 1000 cc. over a flame so adjusted that an hour was required for the contents of the distilling flask to reach the boiling point. With this precaution, air expelled from the apparatus passed through the liquid in the receiver at the rate of a bubble at a time. Approximately 100 cc. of liquid were driven over, the entire distillation taking an hour and a half or more. The eight distillates were then combined and redistilled in a similar manner, the original solution being thereby concentrated to about 115 cc.

A hydroxylamine estimation of this solution gave a recovery of 0.0052 gm. of acetaldehyde from the 0.0055 gm. contained in the original 8000 cc. This agreement is as close as can be expected with hydroxylamine titration, which is only moderately sensitive as a micro method. The distillation process as outlined was therefore assumed efficient and was made the basis of later quantitative experiments.

*Treatment of Blood Samples.*—The operations of collecting, clarifying, and concentrating the ox blood were conducted with two separate objects in view; *viz.*, to inhibit as far as practicable possible changes in the blood—after shedding and before distillation—which might give rise to acetaldehyde not actually present *in vivo*; and conversely, to prevent loss of acetaldehyde normally present in the blood.

The Folin-Wu reagents and water for laking the blood, contained in large bottles were surrounded with finely crushed ice several hours in advance and were practically at 0° upon reaching the abattoir. The blood was laked and precipitated in the killing room in order to reduce to a minimum the time in which post-mortem changes might occur.

The first blood sample was taken from a cow, which had been poleaxed and was bled from the jugular vein within 2 or 3 minutes. The blood was caught with slow stirring in a clean 3 gallon pail to which had been added 50 cc. of half saturated potassium oxalate solution. The blood was measured immediately into the ice-cold water, laked by thorough shaking, and the Folin-Wu reagents were added promptly. The bottles, eighteen in number, each containing 200 cc. of blood precipitated and diluted to 2000 cc., were again packed in ice and returned to the laboratory.

Here twelve 8 inch filter funnels were set up in a cold room over receivers packed in ice. The filters were kept full, and by changing the papers every hour and rejecting the sludge each time, a total of 18 liters of filtrate, representing 1800 cc. of cow blood, were collected within 3 or 4 hours. This volume was then concentrated by distillation as described to about 115 cc.

*Precipitation with Dimedon.*—2 gm. of dimedon (Kahlbaum preparation) were dissolved in 20 cc. of 95 per cent ethyl alcohol. To the 115 cc. concentrate from the final distillation in a large weighing bottle was added 0.5 cc. of the alcoholic dimedon. After 2 days, a white precipitate of fine needles and broken plates was found floating on the surface of the liquid and scattered over the sides and bottom of the bottle. The liquid was poured into a small hardened filter paper, and the filtrate was set aside. The precipitate was transferred to the filter paper and washed free of unchanged dimedon with water. This can be done without loss of aldomecon which, according to Hirsch (14) who used dimedon

for separating acetaldehyde from muscle, is soluble to only 0.0079 per cent at 19°. The crystals were allowed to dry in the air.

The solution separated from the crystals was poured back into the weighing bottle, and 0.5 cc. additional dimedon solution was added. After 2 days, a second crop of crystals appeared of about the same bulk as the first. This was likewise separated, collected on a separate filter paper, washed, and allowed to dry. A third and much smaller crop was obtained, but this was discarded.

Three visits to the abattoir yielded sufficient material for a thorough examination. Blood was taken from a cow on the first trip and from steers on the second and third, representing a total volume of blood of approximately 5.4 liters treated with dimedon. The first crops of crystals from the different runs were collected on the same filter paper, and the second crops were combined on another filter paper. In every case it was found that the aldomedon precipitated satisfactorily without the heating and addition of NaCl utilized by Stepp and Feulgen (8, 10).

Two melting point determinations were made on each crop without purification. The four samples fused completely over less than 2°, thus: first crop, 139.1–140.9°, 139.6–141.4°; second crop, 138.5–140.2°, 138.5–139.9°. Melting point determinations on aldomedon prepared from c.p. acetaldehyde, not recrystallized, were 140.8–141.4°, 140.9–141.5°.

For recrystallization the second crop of crystals from blood was dissolved in 3 cc. of cold alcohol, and the solution was poured with vigorous stirring into 50 cc. of cold water. A granular precipitate formed, which after 2 days was filtered off, washed, and allowed to dry in the air. One sample melted at 138.7–140.0°, and a second at 139.1–140.3°. Samples of the c.p. aldomedon, recrystallized under identical conditions, melted at 139.8–140.6° and 140.3–141.1°.

Mixtures of the c.p. aldomedon and the product from blood melted within the same limits. All thermometer readings are corrected for errors in calibration and due to exposed stem. The melting point of aldeomedon is quoted as 138–140°.

*Anhydride of Aldomedon.*—The identification of acetaldehyde was rendered doubly certain by the preparation of the anhydride, according to the method of Stepp and Feulgen. The first crop of aldomedon from blood was dissolved in 5 cc. of glacial acetic

acid in a test-tube to which a piece of glass tube was then sealed to serve as an air reflux condenser. The solution was heated for 7 hours on a boiling water bath, during which it acquired a yellowish tinge. When cold it was poured into 50 cc. of cold water with stirring. The crystals were subsequently collected on a filter paper, washed with 10 per cent aqueous sodium carbonate to remove unchanged aldomedon, then with water, and finally allowed to dry in the air. The crystals melted at  $175.5\text{--}176.5^{\circ}$  (corrected); c.p. aldomedon anhydride prepared similarly melted at  $175.5\text{--}176.5^{\circ}$  (corrected) also. A mixture of the blood and c.p. products melted at the same temperature. The melting point of the anhydride is given in the literature as  $173\text{--}175^{\circ}$ .

The authors found no characteristic crystal form for aldomedon from any source. By allowing a solution in 80 per cent alcohol to evaporate slowly in the air, perfect rhombs could be obtained, but there were also numerous notched forms of several different types.

The colors of the fused reagent and derivatives are different. Dimedon, aldomedon, and the anhydride are white crystals; after melting, dimedon turns red and the anhydride turns brown, while aldomedon remains white on solidifying.

As the substance from blood melts relatively sharply and behaves precisely as aldomedon, it may be safely concluded that the aldehyde recovered was acetaldehyde without appreciable contamination by other aldehydes.

*Blank Determination.*—On account of the relatively large volume of blood used, it is conceivable that the acetaldehyde actually originated in the filter paper or reagents. To answer this objection, the operations performed on the blood samples were repeated in a blank experiment with every precaution to prevent the escape of possible traces of acetaldehyde in any of the materials. No precipitate was obtained, and the result was also entirely negative after heating the dimedon mixture and adding NaCl. Therefore the aldomedon obtained in the actual experiments could have come only from blood.

## *II. Quantitative. Aldehyde Content of Dog Blood.*

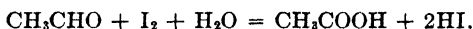
From experiments with dimedon it was evident that most, and possibly all, of the aldehyde content of the blood is acetaldehyde.

Therefore, the amount of acetaldehyde present can be ascertained with tolerable accuracy by calculating as acetaldehyde the volatile reducing material recovered by any general method for determining minute quantities of acetaldehyde in the presence of a large excess of ketone. The procedure introduced by Bougault and Gros (15), and utilized by Fabre (4) and Supniewski (5) for their quantitative work, is the one best suited to the purpose.

*Principle of Method.*—Bougault and Gros placed on a quantitative basis the familiar observation that aldehydes and ketones react with Nessler's solution. In the presence of this reagent, aldehydes are oxidized to corresponding acids and the mercuric salts of the Nessler solution are reduced to a corresponding degree to the mercurous state or to metallic mercury. Ketones form a mercury complex, but this can be broken up by acidifying.

To estimate aldehydes in the presence of ketones, Bougault and Gros treated the solution with Nessler's reagent, acidified, and added an excess of iodine solution. Iodine corresponding to the aldehyde present was utilized; the quantity consumed could be determined by titrating the residual iodine with sodium thio-sulfate.

For purposes of calculation it can be assumed that the aldehyde is oxidized directly by the iodine, as follows:



*Precautions Necessary.*—Both Fabre and Supniewski appear to have followed the directions of Bougault and Gros without considering possible special errors in applying the technique to blood filtrates. Preliminary experiments convinced the present authors that the Nessler method in its original form is not satisfactory for this purpose, and that the data which the two previous workers obtained with it require correction. The method described by Bougault and Gros for estimating "free" acetone adopted by Fabre and by Supniewski as a quantitative one for free acetaldehyde, has been found to be quantitatively unreliable. In demonstrating that the Nessler reagent can be used to determine aldehyde in an aldehyde-ketone mixture, Bougault and Gros reported an experiment in which an equal weight of the interfering substance was added without vitiating the result. But Fabre and Supniewski knew from their own results on the acetaldehyde

content of blood that there was a manifold excess of a possible interfering substance, *viz.* the acetone of normal blood, amounting according to Marriott (16) to 36 mg. per liter in the case of dogs; this source of error was greater in the case of diabetic blood. The writers found that the excess of acetone does affect seriously the determination of acetaldehyde, and that a correction must be applied to all results. For a similar reason, acidification of the Nessler reagent must be decided and controlled, because it was found that the acetone-mercury complex may not break up although the mixture is acid to litmus. An appreciable excess of acid is necessary. Again, on account of the fact that the samples contain much less aldehyde than those with which the originators of the method worked, the percentage error is large unless an iodine solution of minimum working strength is used in place of  $N/10$  concentration. The Nessler solution must be prepared in advance, so that the inevitable precipitate can be removed. Unless this precaution is observed, a precipitate may form in freshly mixed Nessler's reagent sufficient to influence titration with  $N/250$  solution.

*Modified Procedure.*—The Nessler solution selected was that of Sutton (17). The determinations, always in duplicate, were made as follows: pipette 40 cc. of Nessler's reagent for each sample and 40 cc. for each of two blanks into stoppered flasks. Add the samples of solution containing acetaldehyde, and to each of the blanks add an equal volume of water. After 1 hour, cool the flasks containing the mixtures in ice, and keeping them cool, neutralize slowly to litmus paper by adding from a burette 1:3 HCl (1 volume of concentrated acid diluted with 3 volumes of water). Add 1 cc. of the diluted HCl in excess. Next add 20 cc. of iodine solution approximately  $N/250$  and allow the flasks to stand for a 2nd hour with occasional shaking. Then titrate with standardized thiosulfate solution of  $N/250$  concentration using starch indicator. From the average of the titrations of the blanks subtract the titrations of the samples; the difference is the thiosulfate equivalent of the iodine used to reoxidize the Nessler reagent, and it corresponds to the amount of acetaldehyde present. From the equation, 1 cc. of  $N/250$  thiosulfate represents 0.088 mg. of acetaldehyde in the sample. In each determination it is essential to use blanks, exposed for the same length of time and at

the same temperature, and neutralized and titrated in exactly the same manner, as the samples proper.

This modified method was checked against a standard acetaldehyde solution. 2 cc. were used of a solution of acetaldehyde found 0.0213 gm. per 100 cc. by the hydroxylamine sulfate method. The Nessler method gave a recovery of 0.411 mg. on these samples, or a concentration for the original solution of 0.0206 gm. per 100 cc., which is in satisfactory agreement with the hydroxylamine sulfate figure of 0.0213 gm. per 100 cc.

*Ketone Error.*—In order to ascertain the effect of the acetone in normal blood and in diabetic blood on the determinations of acetaldehyde, the attempt was made to recover acetaldehyde from solutions to which acetone and acetaldehyde had been added in the amounts in which they were to be expected in 200 cc. samples of blood. The proportions, which were decided by the determinations of acetaldehyde in the preliminary experiments, were samples equivalent to normal blood, 0.008 gm. of acetone and 0.00076 gm. of acetaldehyde each; samples equivalent to diabetic blood, 0.040 gm. of acetone and 0.00076 gm. of acetaldehyde each. The recoveries by the modified Nessler method were, normal mixture, 0.00057 gm. of acetaldehyde; diabetic mixture, 0.00040 gm. of acetaldehyde. The amount of acetaldehyde added was confirmed by control samples containing acetaldehyde alone. It was thus apparent that the presence of the acetone lowers the result for the acetaldehyde. In the case of a normal blood sample, the apparent aldehyde content as found by the Nessler method is  $\frac{47}{100}$ , i.e.  $\frac{3}{4}$  of the actual value; and in the case of a diabetic blood sample, the apparent value is  $\frac{40}{100}$  or approximately half of the true value.

Several modifications of the method were tested in order to see whether the acetaldehyde could not be recovered completely in the presence of acetone, but the attempts were unsuccessful. Further, it did not appear that the alterations of the directions of Bougault and Gros were responsible for the low results. Nevertheless, the error due to ketone can be gauged, and it was assumed that when proper corrections are made the method is capable of revealing significant changes in blood acetaldehyde, such as the increase to ten times normal value which is reported by Fabre and Supniewski.



*"Free" Acetaldehyde.*—With the probable errors of the Nessler method determined, the free acetaldehyde method of Fabre and Supniewski was inspected. Blood filtrate was boiled to drive off acetone and acetaldehyde; to two samples of 250 cc. in crystallizing dishes was added 0.00022 gm. of acetaldehyde; Nessler's solution was placed in an evaporating dish suspended above the larger vessel, which was then set on a sheet of plate glass; a small bell jar was placed over each set of vessels and they were left for 2 days, together with the usual blanks. At the end of this time the acetaldehyde was determined by the modified Nessler procedure. The recovery was 0.00004 mg. of acetaldehyde, *i.e.* only  $\frac{1}{6}$ . Hence the "contact" method can be regarded as of qualitative significance only, since the recovery in this experiment should have been complete if the free and combined states of acetaldehyde have any significance.

*Routine for Total Acetaldehyde.*—From the foregoing observation, it is evident that only data provided by the direct distillation of blood filtrate can be of significance in the comparison of the normal and diabetic states. In order to guard against the liberation of ammonia or amines during the distillation process which might affect the Nessler solution, the acid part of the Folin-Wu reagent was made by diluting 20 cc. of concentrated  $\text{H}_2\text{SO}_4$  to 1000 cc.; the tungstate part was the usual 10 per cent solution of sodium tungstate dihydrate. These proportions furnished blood filtrate decidedly acid to litmus paper. The precautions previously observed in the preparation of aldomedon for working without loss of time and at as near to freezing point as possible, were observed in the quantitative study. Dogs were used exclusively for the quantitative work.

For taking the blood samples, the animals were stunned, suspended by the hind legs, and bled at once from the carotid arteries and jugular vein. The blood was stirred with about 1 gm. of potassium oxalate as it was caught; dogs of average size yielded from 400 to 900 cc. of blood. It was precipitated immediately in 200 cc. lots in bottles in the special Folin-Wu reagent, ice-cold; afterwards filtration and distillation were carried out according to foregoing directions. The final distillates, amounting to 200 to 400 cc., were mixed together and divided into two samples which were added to the Nessler solution and analyzed in the regular way.

*Recovery of Added Acetaldehyde.*—A final test of the method was performed by analyzing a blood sample to part of which c.p. acetaldehyde had been added in an amount proportionate to that in which it normally occurs. The blood was taken from a large dog ( $\sigma$ ). Two samples of 200 cc. were precipitated and analyzed in the ordinary way. To each of two other 200 cc. samples, still warm, was added 0.00059 gm. of acetaldehyde in aqueous solution, following which these two samples were precipitated and analyzed also. The straight samples of blood analyzed 4.1 mg. per liter; the augmented samples analyzed 5.9 mg. per liter.

The difference, 1.8, is the apparent acetaldehyde added in mg. per liter. It has been shown that this result must be increased to  $\frac{4}{3}$  as a correction for the error due to the acetone present; this gives a corrected result of 2.4 mg. per liter for the added acetaldehyde. Actually there was added per liter  $5 \times 0.00059$  gm., or 2.9 mg., and the analysis is in error  $\frac{1}{6}$ . This recovery was regarded as sufficiently close for the purpose in hand and indicates the working limits of the method.

*Data for Normal Dogs.*—In the preceding experiment the value for the straight blood sample of 4.1 mg. per liter was the apparent blood acetaldehyde of a normal animal. Correcting for acetone error gave the value of 5.5 mg. per liter for the acetaldehyde content of the blood of this dog.

The blood of three other normal dogs was analyzed similarly. The final duplicate thiosulfate titrations for each of the three animals and the calculations are given in Table I. In the final column of this table is given in mg. per liter the acetaldehyde content of the blood, corrected for acetone error.

From the four results of 5.5, 3.8, 3.6, and 2.6 mg. per liter, it was concluded that the acetaldehyde content of the blood of normal dogs is not a constant quantity. The values for dogs reported here were between 2 and 6 mg. of acetaldehyde per liter.

*Depancreatized Dogs.*—Three large dogs were depancreatized. Postoperative recovery was good in every case. After the operation the dogs were given twice daily 150 gm. of meat, 50 gm. of pancreas, 60 gm. of sugar, and 10 units of insulin. The animals remained in healthy condition and were deprived of food and insulin for several days before they were despatched. At the end of the experiment they were weak and emaciated; postmortem

examination indicated complete removal of the pancreas. Ketone bodies were determined in the blood by the method of Van Slyke and Fitz (18), and reducing sugar by the Shaffer-Hartmann method (19).

The acetaldehyde analyses were carried out precisely as for normal dogs; they are given in Table II.

The last column of Table II gives the apparent acetaldehyde values, to which corrections for the ketone error must be applied.

TABLE I.  
*Acetaldehyde Content of Blood of Normal Dogs.*

Sex.	Net thiosulfate used.		Thiosulfate used, average.	Acetaldehyde in sample.	Blood represented.	Acetaldehyde per liter	
	Sample 1.	Sample 2.				Uncorrected.	Corrected.
	cc.	cc.	cc.	mg.	cc.	mg.	mg.
♂	1.79	1.91	1.85	0.16	57	2.9	3.8
♀	5.36	6.04	5.70	0.50	180	2.8	3.6
♀	3.66	3.38	3.52	0.31	150	2.1	2.6

TABLE II.  
*Acetaldehyde Content of Blood of Depancreatized Dogs.*

Sex.	Depancreatized.*	Fasted.*	Blood sugar.	Thiosulfate used, average of two samples.	Acetaldehyde in sample.	Blood represented.	Acetaldehyde per liter.
	days	days	per cent	cc.	mg.	cc.	mg.
♂	14	6	0.43	2.28	0.20	152	1.3
♀	16	3	0.35	3.13	0.28	150	1.8
♀	10	3	0.34	2.94	0.26	138	1.9

\* Before being killed.

Ketone bodies in the samples from the first dog were not determined, but assuming that they were at the usual diabetic level, the result of 1.3 mg. per liter should be doubled to get the true acetaldehyde content of the blood. Ketone bodies were 40 mg. per 100 cc. in the blood of the second animal, and they call for the full correction of  $2 \times$ , giving a corrected result of 3.6 mg. per liter. In the case of the third animal, the ketone bodies were 0.34 mg. per 100 cc., or at the normal level, and hence the correction of  $\frac{1}{2}$  must be applied, giving a corrected result of 2.4 mg. per liter.

Consequently the analyses of the blood of the three dogs de-pancreatized do not indicate any significant change from the level of 2 to 6 mg. per liter found for normal animals.

#### DISCUSSION.

The amount of acetaldehyde which we have shown to be normally present in dog blood is five to ten times as great as the total acetaldehyde of Fabre and Supniewski. In this connection it is instructive to consider the yield of aldome $\dot{d}$ on in the identification experiments. The weight of the crystals was 0.12 gm., representing 0.016 gm. of acetaldehyde, which was recovered from 5.4 liters of blood. Thus each liter of the ox blood contained not less than 3 mg. of acetaldehyde. This is in agreement with our analyses of normal dog blood by the Nessler method.

It will be observed that the analyses recorded in this paper were performed on blood distillates. This raises the question considered by Fabre and Supniewski as to whether the acetaldehyde exists in the free state or in combination. The results of Fabre and Supniewski with the so called free method which we have criticized as being without quantitative significance, should nevertheless prove that at least part of the acetaldehyde exists in the blood uncombined, but when we repeated certain of these experiments the question arose as to whether bacterial action occurred in the blood filtrates, which had to be kept 2 days for the tests. When we suspended Nessler's solution over 5 liters of blood filtrate kept at room temperature, at which Fabre worked, no significant titratable reduction took place; when the same experiment was performed at 37°, the reagent was reduced, but the filtrate was thickly populated with bacterial cells. The addition of 0.08 per cent of optochin, which was used by Supniewski specifically to preserve organ pulp mixtures, did not restrict growth in our blood filtrates, and increasing the optochin concentration to 0.25 per cent inhibited the development of microorganisms to only a slight degree. We found that sulfuric acid proved more satisfactory as an antiseptic. To 1120 cc. of filtrate of blood from a normal dog was added sufficient strong  $H_2SO_4$  to give N/10 concentration in the filtrate; 40 cc. of Nessler's solution were suspended above the liquid in a dish supported on a glass triangle, and the apparatus was covered with a bell jar and placed in an

incubator at 40°. After 2 days the Nessler's solution had absorbed volatile reducing material corresponding to 2.9 mg. per liter of acetaldehyde in the original volume of blood taken of 112 cc. Hence some of the acetaldehyde of the blood exists in either the free state or in some loose combination unstable at 40°.

Our analyses have shown that no pronounced difference in the acetaldehyde content of the blood distinguishes the normal condition from the diabetic state produced by pancreatectomy. Hence acetaldehyde does not appear to play the significant part in deranged metabolism assigned to it by the experiments of Fabre and Supniewski on depancreatized animals, which we have been unable to confirm. Among considerations which have a bearing on the presence of acetaldehyde in the body is the suggestion of Neuberg (13) that amino groups of the blood may possibly serve as physiological fixation agents for acetaldehyde. Again, a mechanism for maintaining the acetaldehyde content below the level at which it becomes toxic is revealed by the work of Battelli and Stern (20) who found aldehyde mutase in various organs of the body, especially the liver, which converts acetaldehyde into ethyl alcohol and acetic acid according to the Cannizzaro reaction.

On the other hand, the investigations of Stepp and Feulgen demonstrate that acetaldehyde cannot be eliminated from the diabetic picture. They were able (8) to recover sufficient acetaldehyde from 5 liters of diabetic urine for positive identification; but apparently 150 liters were necessary for the identification in normal urine (10). If these results imply a decided increase in the acetaldehyde content of the urine in diabetics, then since there is no corresponding increase in blood acetaldehyde, attention is directed to the kidneys for further information respecting the place of acetaldehyde in diabetes.

#### SUMMARY.

1. The presence of acetaldehyde in normal blood has been verified.
2. Normal dog blood contains 2 to 6 mg. of acetaldehyde per liter.
3. There is no significant increase of acetaldehyde in the blood following pancreatectomy.

The authors wish to acknowledge their indebtedness to Professor H. B. Speakman for valuable criticism and advice.

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# THE SPECIFIC ROTATORY POWER OF GLUCOSE-INSULIN SOLUTIONS IN CONTACT WITH MUSCLE TISSUE IN VITRO.

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In a recent series of papers Lundsgaard and Holbøll (1) have reported the effects upon the specific rotatory power of glucose-insulin solutions in contact with muscle tissue *in vitro*. They advance the theory that under the conditions of their experiments there is a fall in the specific rotation of the glucose solution from the usual value,  $52.5^\circ$ , for  $\alpha, \beta$ -glucose to a new value varying from  $22$  to  $40^\circ$ . This form they call new-glucose. In later papers (2, 3, 4, 5) they demonstrate that this new form of glucose is present in various biological fluids, but is absent from the blood of diabetic patients.

The results of these investigations seem to be of considerable importance to our understanding of carbohydrate metabolism. However, Barbour (6) and Paul (7) were unable to confirm the results of Lundsgaard and Holbøll in this connection.

Soon after the appearance of the second series of papers by the latter investigators we began to make a study of the control experiments necessary in an investigation of this nature before attempting to repeat their work on glucose-insulin-muscle solutions.

## *Control Experiments.*

Pfanstiehl's C.P. glucose was used in all cases. Its specific rotatory power varied from  $49.7$  to  $53^\circ$  at equilibrium. The insulin was the commercial product of Eli Lilly and Company, containing 20 units per cc. The collodion sacs were prepared from U.S.P. collodion by filling large test-tubes, draining 5



minutes to make sure that all the ether had escaped, then adding 80 per cent alcohol for 10 minutes, and after removing same placing the sacs in distilled water with a few drops of toluene until ready for use. The bags were then tested for leaks. Preliminary experiments showed that they were impermeable to protein but easily permeable to glucose.

Polariscopic readings were made with a Schmidt and Haensch instrument reading in angular degrees using 2 dm. tubes. As the light source a 75 candle power frosted bulb was used, the rays being filtered through a saturated solution of potassium dichromate. All readings were made at 20°C., and an average of six to sixteen determinations was taken in computing results. The reducing value of the dialysates was determined by the Hagedorn-Jensen method (8) in quadruplicate after diluting the samples so that they contained about 0.1 per cent glucose.

#### *Method.*

200 cc. of a 2 per cent glucose solution, ( $[\alpha]_D^{20}$  49 to 52.3° at equilibrium) in 0.9 per cent NaCl were placed in the warm room at 37°C. for 2 hours, then 50 units of insulin or 15 gm. of fresh muscle tissue added and allowed to remain for 2 hours longer at the same temperature. 25 cc. samples were then removed and dialyzed through collodion tubes against 75 cc. of 0.9 per cent NaCl for 1½ hours. After the reducing and rotatory powers were determined the samples were removed and the dialysates allowed to stand at room temperature for 24 hours for further study.

In a total of 60 determinations with the glucose-insulin solutions we found very low values in 9 cases, whereas in the remaining experiments the variations in the specific rotatory power were within experimental error. No change was observed in the glucose-muscle solutions.

As these studies were in progress there appeared the papers of Barbour (6) and Paul (7), both of whom were unable to observe any discrepancy in the reducing and rotatory power of glucose solutions under various conditions. The former investigator used muscle tissue from various animals, while the latter studied both the dialysates and ultrafiltrates from the glucose solutions.

Our next problem was to study the effects upon the specific rotatory power of the sugar solutions when both muscle tissue

and insulin were added. We used the same methods as described above.

## EXPERIMENTAL.

962.5 cc. of 2, 4, or 6 per cent glucose in 0.9 per cent NaCl were placed in the cold room for 24 hours, then at room tem-

TABLE I.

*Specific Rotatory Power of Glucose-Insulin Solutions on Contact with Muscle Tissue.*

Original concentration.	Glucose in dialysate.		Zero reading.	Reading at equilibrium.		$[\alpha]_D^{20}$	Reading after 1½ hrs. dialysis.		$[\alpha]_D^{20}$
	Reduction.	Rotation.		Observed.	Corrected.		Observed.	Corrected.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
<i>per cent</i>									
2.00	0.47	0.44*	0.36°	2.43°	2.07°	51.7°	0.82°	0.46°	49.0°†
2.00	0.47	0.43	0.36°	2.43°	2.07°	51.7°	0.80°	0.44°	46.0°
2.00	0.47	0.44	0.36°	2.43°	2.07°	51.7°	0.81°	0.45°	47.5°
2.00	0.46	0.38	0.36°	2.43°	2.07°	51.7°	0.75°	0.39°	42.4°
2.00	0.47	0.46	0.36°	2.43°	2.07°	51.7°	0.84°	0.48°	51.0°
2.00	0.48	0.46	0.36°	2.37°	2.01°	50.2°	0.82°	0.46°	48.0°
2.00	0.49	0.50	0.36°	2.37°	2.01°	50.2°	0.86°	0.50°	51.2°
2.00	0.48	0.48	0.36°	2.37°	2.01°	50.2°	0.84°	0.48°	50.3°
2.00	0.47	0.40	0.36°	2.37°	2.01°	50.2°	0.77°	0.41°	42.1°
4.00	0.97	0.94	0.35°	4.53°	4.18°	52.3°	1.34°	0.99°	50.8°
4.00	0.98	1.02	0.35°	4.53°	4.18°	52.3°	1.41°	1.06°	53.6°
4.00	0.98	1.02	0.35°	4.53°	4.18°	52.3°	1.43°	1.07°	54.4°
6.00	1.45	1.38	0.35°	6.59°	6.24°	52.1°	1.79°	1.44°	49.7°
6.00	1.43	1.41	0.35°	6.59°	6.24°	52.1°	1.82°	1.47°	51.4°
6.00	1.40	1.41	0.35°	6.59°	6.24°	52.1°	1.82°	1.47°	51.4°

$$51.7 - \frac{100 \times 0.46}{2 \times C} = 0.444.$$

$$\dagger [\alpha]_D^{20} = \frac{100 \times 0.46}{2 \times 0.47} = 49^\circ$$

perature for 5 hours. At equilibrium the specific rotatory power was determined. In some experiments 25 cc. of phosphate buffer mixtures of pH 7.38 were added, while no buffers were used in other cases. The solutions were placed in the warm room at 37°C. for 1 hour; then 250 units of insulin and 75 gm. of fresh muscle tissue added. The mixtures were allowed to remain at 37°C. for 2 hours with continual shaking.

The animals, rats or rabbits, were killed by a blow on the back of the head, and the muscle tissue removed and placed in the solution. This operation usually required about 10 minutes.

After 2 hours incubation, 25 cc. samples were dialyzed through the specially prepared collodion tubes into 75 cc. of 0.9 per cent NaCl. The height of the liquids on both sides of the membrane was the same. The specific rotatory power of the solutions was determined (a) after equilibrium was reached, (b) after warming at 37°C. for 2 hours, and (c) after dialysis at room temperature for 1½ hours. The reducing power was determined by the Hagedorn-Jensen method (8) in quadruplicate.

A total of 15 experiments was performed. We did not vary the conditions, except in one instance, as we were primarily interested in proving the presence or absence of new-glucose in the dialysates by means of its low specific rotatory power. A protocol of one typical experiment is given in Table I.

#### DISCUSSION.

An examination of the table shows that the same small errors occur here as in the case of the glucose-insulin solutions. It seems to us that the chief source of error lies in the *small difference* between the zero and observed readings. Using the dialysates from 2 per cent glucose solutions, we obtained readings varying from 0.75 to 0.84°; then, after deducting the zero reading, 0.35°, we obtained as our final value 0.40 to 0.49°. Again, we found that a difference of 0.01° in the observed reading would correspond to a variation of 0.7° in the specific rotatory power and this is the cause of the lowered values in Column 10.

There are also very small differences between the concentration of glucose calculated from reduction and rotation. Lunds-gaard and Holbøll (9) in their calculations of the latter value assume that their glucose had a specific rotation of 52.5°. This is true only if they used the purest sugar obtainable. We determined this value for our solutions before each experiment and found that it varied from 49 to 53°; hence we used these actually determined values (Column 7) in calculating the concentration by rotation (Column 3). The small differences between the reducing and rotating figures, therefore, are due to experimental error and not to the combined action of insulin and a substance from muscle tissue upon the glucose molecule.

In order to eliminate these variations we also used 4 and 6 per cent glucose solutions, and here the discrepancies between the reducing and rotatory values, and also between the specific rotatory powers, are small and are well within experimental error.

The chief point of interest in these investigations is that we did not observe a specific rotatory power of the glucose-insulin-muscle solutions below  $42^{\circ}$ . In no case was a value of 22 to  $40^{\circ}$ , corresponding to new-glucose, obtained.

The dialysates were allowed to stand at room temperature for a period of 24 hours, and in every case without exception they were so cloudy that it was impossible to get a reading on the polariscope.

#### CONCLUSIONS.

1. The specific rotatory power of glucose-insulin solutions in contact with fresh muscle tissue is only slightly lower than the usual value,  $+52.5^{\circ}$ . These variations are due to experimental error. With the use of larger concentrations of glucose, the reducing and rotatory values and also the specific rotatory powers agree closely.

2. We have been unable to confirm the results of Lundsgaard and Holbøll as to the production of new-glucose *in vitro* from the glucose-insulin-muscle solutions.

3. The results obtained are in close agreement with those of Barbour and Paul.

We wish to thank Eli Lilly and Company for the supply of insulin used in these investigations.

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# A METHOD FOR THE DETERMINATION OF ALLANTOIN IN RABBIT URINE.

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## INTRODUCTION.

During the past 10 years, the methods for the determination of uric acid have been gradually improved, so that short, accurate methods are now available for the estimation of this important catabolite. On the other hand, during the same period, there have been no marked improvements in the methods for allantoin, which are long, tedious, and probably not accurate, especially for small amounts of allantoin. As a consequence, most of the recent studies on purine metabolism have been made on human subjects, who excrete uric acid as the principal end-product of purine metabolism, rather than on those mammals in which allantoin is assumed to have a metabolic significance similar to that of uric acid.

One group of methods for the estimation of allantoin depends upon the precipitation of allantoin from solution as a metallic salt. The methods of this type, which were published prior to that of Wiechowski (1), in 1909, may be dismissed without further consideration, since they have been found to be unreliable. The method of Wiechowski, which is the standard method at the present time, depends on the fact that if urine is diluted so that the urea concentration is 1 per cent or less, and is freed from chlorides and substances precipitated by phosphotungstic acid and basic lead acetate, the allantoin may be completely precipitated by mercuric acetate in the presence of a large amount of sodium acetate. The allantoin may be isolated and determined gravimetrically, or the nitrogen content of the mercury allantoin compound may be estimated. Handovsky (2), in 1914, modified the latter part of the procedure recommended by Wiechowski by the precipitation of the allantoin with a measured amount of a standard mercuric acetate-sodium acetate solution. After removal of the mercury allantoin salt by filtration, the excess of mercuric acetate in the filtrate is determined by titration with standard ammonium thiocyanate.

The second group of methods is based on the fact that, under definite conditions, the allantoin nitrogen as well as the urea nitrogen of a urine may be hydrolyzed to ammonia. A separate determination of the pre-

formed ammonia and the ammonia resulting from the hydrolysis of urea enables one to calculate the allantoin by difference. In the early procedure of Folin (3) for urea, the urine was subjected to the action of hydrochloric acid in the presence of magnesium chloride, and the ammonia formed was considered to have come from urea. Benedict (4) carefully studied this reaction and concluded that in addition to the urea nitrogen, all of the allantoin nitrogen, 2 per cent of the uric acid nitrogen, and 1 per cent of the creatinine nitrogen is converted to ammonia. Plimmer and Skelton (5), in 1914, apparently disregarding the small amount of nitrogen resulting from the breakdown of uric acid and creatinine, followed the procedure of Folin for urea, and considered the ammonia nitrogen to be derived from preformed ammonia, urea, and allantoin. A separate determination of the urea and preformed ammonia was made by the urease method and the allantoin calculated by difference. Harding and Young (6) have simplified the method of Plimmer and Skelton by carrying out the hydrolysis with hydrochloric acid and magnesium chloride, in an Erlenmeyer flask fitted with a reflux tube.

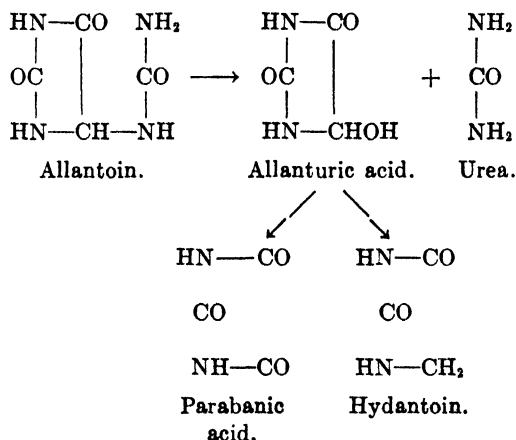
During the course of the last 5 years the writer has made numerous determinations by the Harding and Young method and by the Handovsky modification of the Wiechowski method. It is felt that neither of these methods is satisfactory, especially if a urine contains small amounts of allantoin and normal or high amounts of the other urinary nitrogenous constituents. Since in the method of Harding and Young the allantoin is determined by difference, a small error in the urea determination makes a considerable difference in the value for allantoin. A second objection is the difficulty in exactly duplicating the conditions for the hydrolysis, even though the directions of Harding and Young are followed as closely as possible. It was often observed that the hydrolysis of four samples of the same urine would yield two pairs of checks, although the hydrolyses were carried out carefully under as nearly identical conditions as possible. Although the discrepancy between the pairs was not great, it was enough to cast doubt upon the accuracy of the method.

The titration of mercuric salts by ammonium thiocyanate involves a very difficult end-point, and this fact aside from the length of time required to carry out the determination constitutes a serious objection to the Handovsky method. It is unfortunate that small differences in the final titration with thiocyanate should make considerable variation in the value for allantoin. In our hands, satisfactory recoveries of allantoin

added to rabbit urine could not be consistently obtained by the method of Handovsky. In view of these facts, a direct method for allantoin, which is shorter than the methods of Handovsky and Wiechowski and which will more accurately measure the allantoin content of biological fluids, is desirable. The method to be discussed in this paper, which, it is believed, will meet these qualifications, depends upon the hydrolysis of allantoin to oxalic acid, the precipitation of the latter as calcium oxalate, and its estimation by a permanganate titration.

*Theory of the Determination.*

As early as 1864, Baeyer (7) pointed out that the hydrolysis of allantoin with barium hydroxide yielded oxalic acid as one of the end-products. Claus (8) in 1874 came to the same conclusion, but differed somewhat from Baeyer with respect to the other products of the reaction. According to Baeyer, the alkaline decomposition of allantoin proceeds as follows:



The allanturic acid which is formed undergoes a simultaneous oxidation and reduction to hydantoic and parabanic acid. The hydantoin is further hydrolyzed to hydantoic acid while parabanic acid is converted to oxalic acid and urea.

Thus, according to Baeyer, 2 molecules of allantoin yield 1 molecule of oxalic acid. Claus, however, considers that 3 molecules of allantoin yield 2 molecules of oxalic.



It is the purpose of this paper to present evidence which demonstrates that the hydrolysis of allantoin to oxalic acid may be utilized for the quantitative estimation of allantoin in urine. The method as applied to urine will first be given in detail, in order that the experiments, which it is believed show the principles of the determination to be sound, may be better understood.

### *Method for Urine.*

Since urine is highly pigmented, and may contain varying amounts of oxalic acid, the direct application to the urine of the hydrolysis procedure for the conversion of allantoin to oxalic acid will not yield accurate results. Allantoin forms, under the proper conditions, an extremely insoluble compound with mercuric acetate, but the addition of mercuric acetate to a urine containing allantoin will not result in the quantitative removal of the latter, since the presence of chlorides, ammonia, and high concentrations of urea interfere with this precipitation. Evidence will be presented in this paper, which demonstrates that treatment of the urine with phosphotungstic acid, followed by the addition of basic lead acetate, will remove most of the interfering substances, and will give a solution favorable for the precipitation of the mercury-allantoin compound. After precipitation, the mercury-allantoin compound is decomposed with hydrogen sulfide and the mercuric sulfide removed. The filtrate containing the allantoin is hydrolyzed with sodium hydroxide and the oxalic acid which is formed is precipitated as calcium oxalate. The estimation is completed by the titration of the calcium oxalate with potassium permanganate in the usual manner. The procedure in detail, as it has been applied to the determination of allantoin in rabbit urine, is as follows: The urine is diluted, so that the concentration of the urea is approximately 0.5 per cent. As a rule, the dilution of the 24 hour sample of a rabbit urine to 300 or 350 cc. is satisfactory. Concentrations of urea greater than 0.5 per cent prevent a quantitative precipitation of the allantoin by the mercuric acetate in a later step of the procedure. For the removal of basic compounds, such as uric acid, creatinine, ammonia, and most of the urinary pigments, the urine is acidified and treated with phosphotungstic acid. An acid mixture, consisting of equal parts by volume of glacial acetic acid and 4 N sulfuric acid, is added to an aliquot of the urine in

such an amount that the final concentrations of the acetic and sulfuric acids are approximately 5 and 1 per cent respectively. Since it is extremely important that an excess of phosphotungstic acid be avoided, preliminary tests<sup>1</sup> are made on small portions of urine, and then, on the basis of these tests, the calculated amount of solid phosphotungstic acid is added to the acidified aliquot of urine to be used in the determination. It has been found convenient to use 100 cc. of urine, which are measured into a 150 cc. Erlenmeyer flask and then acidified with 10 cc. of the acid mixture. After the addition of the calculated amount of solid phosphotungstic acid, a rubber stopper is inserted and the flask vigorously shaken to insure the complete solution of the phosphotungstic acid.

After 1 hour the precipitate is removed by filtration and solid lead oxide (PbO) is added, until the solution reacts faintly alkaline, due to the formation of basic lead acetate by the interaction of the lead oxide with the sulfuric and acetic acids. Usually 16 to 19 gm. of lead oxide are required for this neutralization, and since an excess of the lead oxide is to be avoided, the smaller amount is first added to the filtrate, which is preferably contained in a 150 cc. Erlenmeyer flask. The flask is gently rotated until the yellow lead oxide has been replaced by the white lead salts, and the flask has become appreciably warmer, due to the heat of neutralization. A rubber stopper is now inserted and the flask vigorously shaken. A small portion (2 cc.) is filtered into a test-tube and a drop of phenol red<sup>2</sup> indicator added. If the color

<sup>1</sup> To determine the correct amount of phosphotungstic acid required to precipitate the basic substances, 2 cc. portions of urine are measured into test-tubes and acidified with 0.2 cc. of the acid mixture (equal parts of glacial acetic and 4 N sulfuric acids). Measured portions of a 10 per cent aqueous solution of phosphotungstic acid are now added from a Mohr pipette to the test-tubes containing the acidified urine. At the end of 5 minutes, the contents of the tube are filtered, and small amounts (0.2 cc.) of the 10 per cent phosphotungstic acid solution are added. Those tubes in which no further precipitate is obtained in 3 minutes are considered to have had sufficient phosphotungstic acid for the removal of the basic compounds. From this preliminary test one calculates the smallest amount of solid phosphotungstic acid required for the desired aliquot of urine.

<sup>2</sup> The phenol red and brom-cresol purple indicators were made according to the directions given in Clark's "Determination of Hydrogen Ions," and a rough estimation of the pH was made by matching the colors of the solutions with the Clark color chart.

indicates that the pH of the solution is lower than 7.2, a drop of brom-cresol purple is added to the same tube. If a pH between 6.0 and 7.0 is indicated, it is necessary to add only a small amount of the lead oxide to the major portion of the solution to bring it to a pH of 7.2. Phosphates, sulfates, some of the urinary pigments, preformed oxalic acid, and any excess of phosphotungstic acid are precipitated by the basic lead acetate as insoluble lead salts. To obtain the greatest quantity of filtrate from the bulky lead precipitate, it is advisable to transfer the entire mixture to 50 cc. centrifuge tubes and throw out the major portion of the precipitate by centrifugation. The supernatant liquid is filtered through a small filter and a clear solution with a faint green tinge is obtained. Aliquot portions of this filtrate, which should have a pH of 7.2 to 7.4, are measured into 50 cc. centrifuge tubes, and a solution of 1 per cent mercuric acetate in 30 per cent sodium acetate is added. The final concentration of the mercuric acetate should not fall below 0.2 per cent. As a rule, if 100 cc. of urine are used for the analysis, the volume of the filtrate following the treatment with lead oxide is such that two 40 cc. portions may be taken for analysis. For this amount of filtrate, 10 cc. of the mercuric acetate-sodium acetate reagent are added. The mercuric acetate is thoroughly mixed with the solution by means of a stirring rod, and the tubes are set aside for  $\frac{1}{2}$  hour. At the end of this time, the precipitates<sup>3</sup> are removed by centrifugation. If it is not convenient to centrifuge at the end of  $\frac{1}{2}$  hour, the period of precipitation may be extended to 1 hour, since numerous experiments have demonstrated that the differences in results obtained with a precipitation period of  $\frac{1}{2}$  hour, as compared to 1 hour, are within experimental error. The supernatant liquids<sup>4</sup> are discarded and the precipitates are

<sup>3</sup> The precipitate obtained when the mercuric acetate-sodium acetate solution is added to the filtrate, which is slightly alkaline because of the presence of basic lead acetate, is not composed entirely of the mercury allantoin compound. If 100 cc. of water are acidified with the acid mixture, neutralized with lead oxide, and this filtrate treated with the mercuric acetate-sodium acetate reagent, a similar precipitate is obtained, although allantoin is not present.

<sup>4</sup> If the conditions are favorable for the precipitation of the allantoin, a portion of this supernatant liquid should give an immediate turbidity with 0.5 mg. of allantoin.

washed in the centrifuge tubes with 15 cc. of distilled water and centrifuged again. The supernatant liquids are again discarded and the precipitates are suspended in 15 cc. of water. 0.2 cc. of concentrated hydrochloric acid is added to each tube. With the use of a stirring rod to break up the larger particles, the greater part of the precipitate is dissolved, with the exception of a small residue of lead chloride. Hydrogen sulfide is now passed into the tubes until the heavy metals have been completely precipitated as sulfides. These are removed by centrifugation and the supernatant liquids which contain the allantoin are decanted into 150 cc. Erlenmeyer flasks, in which the hydrolysis of allantoin to oxalic acid is to be made. The precipitated sulfides are washed in the centrifuge tubes with 10 cc. of distilled water, and the washings added to the Erlenmeyer flasks. The excess of hydrogen sulfide is removed by aeration.<sup>5</sup>

The solutions containing the allantoin are made slightly alkaline by the addition of 1 cc. of a solution of sodium hydroxide<sup>6</sup> which contains 0.5 gm. of sodium hydroxide per cc. of solution. A glass bead is now added to each flask and the flasks are heated over a Bunsen burner until the volume has been reduced to about 12 cc. 9.25 cc. of the same strong alkaline solution are added to each flask and the volume made to 25 cc. with distilled water.<sup>7</sup>

<sup>5</sup> The excess of hydrogen sulfide is removed by aeration rather than by boiling, since heating the solution in the presence of the acid causes marked pigmentation.

<sup>6</sup> 500 gm. of sodium hydroxide are dissolved in water and after cooling made to a volume of 1000 cc.

<sup>7</sup> The correct estimation of this volume in a 150 cc. Erlenmeyer flask is rather difficult. A very close approximation to this volume can be made by adding 25 cc. of water to the dry flask to be used in the hydrolysis, placing it on a level surface and marking the water level with two strips of a label on opposite sides of the flask. By the use of this method, it is possible to gauge the volume to within 2 cc. of the desired amount. A second method is as follows: Add 25 cc. of water to a large number of 150 cc. Erlenmeyer flasks and then prepare a T tube of glass rodding of such a size that when the short arm is placed across the neck of the flask, the long arm will just touch the surface of the water. Better results will be obtained if the tip of the T tube is coated with high melting paraffin. Considerable time may be spent in the selection of a group of flasks which will meet this requirement, but since the same flasks may be used many times for the hydrolysis procedure, it is effort well spent. By following the second procedure, volumes slightly lower than 25 cc. are more likely

The amount of alkali which is added is sufficient to neutralize the small amount of acid present and to make the final concentration of the sodium hydroxide 20 per cent. The contents of the flasks are now heated over a micro burner for 1 hour. Later experiments will demonstrate that the hydrolysis of allantoin for 1 hour at the boiling temperature of 20 per cent sodium hydroxide yields an amount of oxalic acid which is in quantitative agreement with that demanded by Baeyer's theory of the reaction. It is essential for complete hydrolysis that vigorous boiling be maintained during the entire hour.

In order to keep the volume constant during the hydrolysis period each flask is fitted with a test-tube, which serves as a Hopkins condenser. The test-tubes, which should fit snugly but not tightly into the necks of the Erlenmeyer flasks, extend to within 1 inch of the surface of the liquid during the hydrolysis. A small stream of water sent through the condensers will prevent the loss of liquid from the flasks.

It has been our experience that there is very little spattering within the flask, but after the period of hydrolysis it is advisable to wash the portion of the condenser within the flask with a fine jet of water. The flasks are then cooled, 2 drops of methyl red added, and concentrated hydrochloric acid slowly run in from a burette with constant shaking, until the solution is definitely pink. 0.5 cc. of concentrated ammonium hydroxide is then added. It has been found that this treatment is the most efficient for the removal of the siliceous material that has resulted from the action of the alkali upon the glass. The contents of the flask are now transferred quantitatively to a 50 cc. volumetric flask and, after cooling to 20°C., made to volume with distilled water. After a thorough mixing, the contents of the flask are transferred to 50 cc. centrifuge tubes and the precipitate of siliceous material

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to result than volumes over 25 cc. Unless large errors in the estimation of the volume are made, the results will not be vitiated, since the hydrolysis of allantoin with 25 per cent sodium hydroxide yields practically the same results as the hydrolysis by 20 per cent sodium hydroxide. However, if error in the estimation of the volume is such that the concentration of the alkali during the hydrolysis is only 16 per cent, results about 10 per cent lower than theoretical are obtained. (See later experiment on pure solutions of allantoin.)

removed by centrifugation. Aliquot portions (usually 40 to 45 cc.) are measured into 50 cc. centrifuge tubes,<sup>8</sup> and the pH adjusted to 5.8 to 6.0 by the addition of acetic acid. 2 cc. of a 20 per cent solution of calcium chloride are added to each tube, and the inside surface of the tube rubbed with a rubber tipped stirring rod until a fine crystalline precipitate of calcium oxalate is formed. The tubes are set aside for 1 hour and the calcium oxalate is thrown down by centrifuging for 5 minutes at 1500 R.P.M. The precipitate is washed once with 15 cc. of a cold saturated solution of calcium sulfate, which contains just enough ammonium hydroxide to make it faintly alkaline to phenolphthalein, and centrifuged. The washed precipitate is dissolved in 20 cc. of *N* sulfuric acid and titrated with a standard solution of potassium permanganate. The usual precautions for making a permanganate titration are observed. Clark (9) has discussed permanganate titrations of oxalic acid in detail, including the preparation and standardization of weak permanganate solutions.

The tube containing the oxalic acid to be titrated is placed in a boiling water bath, and a small thermometer inserted. When the contents of the tube are heated to 80° C., the tube is removed and the titration made. During the titration the tube is partially submerged in water kept at 90°C. in order that the temperature within the tube shall not fall below 65°C. before the completion of the titration. The hot water is conveniently held in a large porcelain casserole, which serves also to provide a white background for the titration. The permanganate solution should be measured from a burette graduated to 0.02 cc. The amount of the standard permanganate solution required to give 20 cc. of the *N* sulfuric acid the faint pink end-point, is subtracted from all titrations. The permanganate solutions which have been used in this study have varied in strength from 0.015 *N* to 0.019 *N*. If by hydrolysis, 2 molecules of allantoin yield 1 molecule of oxalic acid, 1 cc. of 0.01 *N* potassium permanganate is equivalent to 1.581 mg. of allantoin.

In Table I are recorded the results of the analysis of rabbit urine for allantoin, before and after the addition of allantoin. Recoveries of allantoin ranging from 90 to 104 per cent have

<sup>8</sup> For the precipitation of the oxalic acid, it is advisable to use centrifuge tubes of the type recommended by Clark (9).

been obtained, with an average recovery of 98 per cent. Satisfactory recoveries of added allantoin are obtained only if an excess of phosphotungstic acid and lead oxide is avoided.

Rabbit urines have also been analyzed before and after the addition of egg albumin and glucose. The presence of albumin to the extent of 0.3 per cent was without effect upon the values obtained for allantoin. The influence of higher concentrations of albumin were not studied, since it is possible to remove the larger amounts by heat coagulation, prior to the analysis for allantoin. If the content of glucose in a urine is not more than 0.5 per cent, the values for allantoin are unaffected. Higher

TABLE I.  
*Recovery of Allantoin Added to Rabbit Urine.*

Allantoin per 100 cc. of urine.	Allantoin added to 100 cc. of urine.	Total allantoin found per 100 cc. of urine.	Allantoin recovered.	
mg.	mg.	mg.	mg.	per cent
40.5*	5.0	45.3*	4.8	96
23.2	10.0	33.6	10.4	104
10.9	15.0	24.4	13.5	90
34.7	22.0	56.8	22.1	100
31.0	24.0	55.5	24.5	102
32.5	25.0	57.2	24.7	99
28.4	25.0	51.0	22.6	90
34.5	37.5	70.0	35.5	95
44.5	50.0	96.5	52.0	104

\* The figures given in Columns 1 and 3 are the averages of closely agreeing check determinations.

concentrations of glucose (0.7 to 1.0 per cent) give values for allantoin which are from 4 to 9 per cent lower than those obtained on the original urine.

Experiments have also been made which demonstrate that if the urea concentration of the urine is approximately 0.5 per cent, further dilution of the urine will not lead to higher results for allantoin. A typical experiment may be cited. 100 cc. of a urine containing approximately 550 mg. of urea were analyzed for allantoin as previously described. A 75 cc. and a 50 cc. portion of the same urine were diluted with water to 100 cc. and analyzed in the same manner. The values obtained for allantoin

after calculation to 100 cc. of the original urine were 28.4, 28.1, and 28.9 mg., respectively. The greatest variation, it will be noted, is less than 3 per cent.

Rabbit urines have been analyzed both by this method and by the method of Handovsky. The method proposed in this paper gives values for allantoin which are 15 to 20 per cent higher than those obtained by the procedure of Handovsky. In the Handovsky method, prior to the precipitation of the allantoin as the mercury salt, it is necessary to remove the metallic ions of silver and lead from solution as the sulfides. The precipitate of the sulfides is very bulky, and since this precipitate is not washed, it is thought that considerable loss in allantoin may occur at this step of the procedure. In the new method that is proposed, the allantoin is precipitated without the removal of the lead ions from the solution. The precipitate is decomposed with hydrogen sulfide and the resulting precipitate of metallic sulfides, which is small in amount, is washed with water. It has been our experience that the recoveries of allantoin added to rabbit urine were consistently lower by the Handovsky method, than by the method described in this paper. While the recovery of added allantoin is not a final proof of the accuracy of the method, it is logical to assume, that of the two methods, the one which gives the higher value for allantoin in the original urine and also leads to a higher percentage recovery of the added allantoin is more nearly correct.

As an illustration of the exact procedure, the following experiment is given in detail: A 24 hour sample of rabbit urine was diluted to 350 cc. A 100 cc. portion of this urine was acidified with 10 cc. of the acid mixture and treated with phosphotungstic acid and lead oxide as previously directed. Two 40 cc. aliquots of the filtrate, which were obtained after the lead oxide treatment, were measured into centrifuge tubes and the allantoin precipitated. After hydrolysis of the allantoin, each solution was made to 50 cc. for the removal of silicates and then 45 cc. aliquots carefully measured for the precipitation of the oxalic acid. The titrations with 0.01639 N potassium permanganate for the two check determinations were 3.0 and 3.1 cc. A blank of 0.05 cc., which represents the amount of the permanganate required to give a pink tinge to 20 cc. of N sulfuric acid, is subtracted from the average of these two titrations. Since 3.0 cc. of 0.01639 N



potassium permanganate represent the allantoin of  $\frac{18}{55} \left( \frac{40}{110} \times \frac{45}{50} \right)$  of the original 100 cc. of urine,  $3.0 \times \frac{55}{18}$  or 9.17 cc. represent the allantoin content of 100 cc. of the urine. Since 1 cc. of 0.01639 N potassium permanganate is equivalent to 2.59 mg. of allantoin, the allantoin content of 100 cc. of the urine is 23.75 mg.

*Experiments on Solutions Containing Known Amounts of Allantoin.*

Before an attempt was made to apply the method to urine, numerous experiments were made with pure solutions of allantoin and mixtures of allantoin with other urinary constituents. Solutions of allantoin were hydrolyzed by various concentrations of alkali for periods ranging from 30 to 120 minutes, and the oxalic acid, which resulted from this hydrolysis, was determined in the manner outlined in the method. For amounts of allantoin over 6 mg., the total volume of the hydrolysis mixture was 25 cc., while for amounts less than 6 mg., the hydrolysis was made in a volume of 12.5 cc. In the procedure for the removal of the siliceous material, these volumes were diluted to 50 and 25 cc. and 40 and 20 cc. aliquots respectively were used for the precipitation of the oxalic acid as calcium oxalate. The results of the alkaline decomposition of pure allantoin are given in Table II. In all cases the temperature of the reaction was the boiling point of the alkali employed in the hydrolysis. The amounts of allantoin in Column 4 of Table II are calculated from the amount of oxalic acid found in the reaction mixture, on the assumption that 2 molecules of allantoin yield 1 molecule of oxalic acid. In this table, as well as in succeeding tables, the results are the average of closely agreeing check determinations.

The results given in Table II indicate that the hydrolysis of allantoin by a boiling solution of 20 per cent sodium hydroxide for a period of 1 hour yields an amount of oxalic acid which is in quantitative agreement with that demanded by Baeyer's theory of the reaction. Apparently for concentrations of alkali stronger than 20 per cent, the course of the reaction is somewhat different, since recoveries of allantoin greater than 100 per cent are obtained. If the concentration of sodium hydroxide is 20 per cent or higher, periods of hydrolysis longer than 1 hour also lead to

results higher than theoretical. It cannot be definitely stated that when allantoin is hydrolyzed by 20 per cent sodium hydroxide for a period of 1 hour, that the products of hydrolysis are limited to oxalic acid, hydantoic acid, carbon dioxide, and ammonia. Quantitative recoveries of allantoin are obtained, however, if Baeyer's theory of the reaction is assumed to be correct.

TABLE II.  
*Determination of Allantoin in Pure Solutions.*

Concentration of sodium hydroxide.	Time of hydrolysis.	Allantoin.		
		Present.	Recovered.	
<i>per cent</i>	<i>min.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
16	30	10.0	7.6	76
16	60	10.0	9.0	90
16	120	10.0	9.8	98
16	120	10.0	10.1	101
20	30	10.0	8.9	89
20	60	3.0	3.0	100
20	60	4.0	4.0	100
20	60	5.0	5.0	100
20	60	6.0	6.2	102
20	60	10.0	9.9	99
20	60	10.0	10.0	100
20	60	20.0	20.4	102
20	60	35.0	35.4	101
20	90	10.0	10.0	100
20	120	10.0	10.5	105
25	60	10.0	10.1	101
25	120	10.0	10.3	103
33	60	10.0	10.8	108
33	120	10.0	11.2	112
33	120	10.0	11.6	116

It will be recalled that one step in the procedure for the determination of allantoin in urine is its precipitation as the mercury salt, which is removed by centrifugation. The precipitate is dissolved in weak hydrochloric acid and then decomposed with hydrogen sulfide. After removal of the mercuric sulfide the filtrate containing the allantoin is hydrolyzed with 20 per cent sodium hydroxide. Table III is a record of experiments with

pure allantoin solutions which have been precipitated by mercuric acetate and then treated precisely as outlined in the method for urine. In these experiments the final concentration of the mercuric acetate has varied from 2.5 to 0.25 per cent. The lower concentrations are apparently as efficient for the precipitation of the allantoin as the higher concentrations, providing there is an excess of mercuric ions. It is essential, however, that whatever strength of mercuric acetate is employed, the acidity due to its hydrolysis should be buffered by sodium acetate, so that the precipitating reagent is neutral. In most of our later experi-

TABLE III.

*Recovery of Allantoin after Its Precipitation from a Pure Solution as the Mercury Salt.*

The mercury allantoin compound was decomposed with hydrogen sulfide, and the mercury-free filtrate hydrolyzed with sodium hydroxide.

Concentration of sodium hydroxide.	Time of hydrolysis.	Allantoin.		
		Present.	Recovered.	
<i>per cent</i>	<i>min.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
20	60	3.0	2.8	93
20	60	4.0	3.8	95
20	60	5.0	4.7	94
20	60	5.0	5.0	100
20	60	6.0	5.8	97
20	60	10.0	9.8	98
20	60	10.0	9.7	97
20	60	10.0	9.8	98
20	60	15.0	15.1	100

ments, a solution of 1 per cent mercuric acetate in 30 per cent sodium acetate was used. Since the recoveries in the experiments recorded in Table III are of the same order as those of Table II, it is evident that the precipitation of the allantoin as the mercury salt, and the subsequent removal of the mercuric ions prior to hydrolysis, has not resulted in the loss of allantoin.

In the method of Wiechowski (1) for allantoin, it is stated that correct results cannot be obtained unless the urine to be analyzed is so diluted that the concentration of urea is not more than 1 per cent. He has also pointed out the necessity for the complete removal of the chlorides. The effect of the addition of the urea,

sodium chloride, creatine, and creatinine on the recovery of allantoin from aqueous solutions has been studied and the results have been summarized in Table IV. In each experiment recorded in Table IV, the solution containing the allantoin and the added constituent was adjusted to a pH of 7.2 to 7.4 by the precipitating reagent (1 per cent mercuric acetate in 30 per cent sodium acetate). In these experiments, the mercury allantoin compound

TABLE IV.

*Effect of the Presence of Some Common Urinary Constituents upon the Recovery of Allantoin from Mixtures of Pure Compounds.*

The allantoin was precipitated in every case as the mercury salt, which was then decomposed with hydrogen sulfide, and the mercury free filtrate hydrolyzed for 1 hour with 20 per cent sodium hydroxide.

Concentration of sodium chloride.	Concentration of urea.	Creatine present.	Creatinine present.	Allantoin.		
				Present.	Recovered.	
per cent	per cent	mg.	mg.	mg.	mg.	per cent
0.1*	*			10.0	7.4	74
0.2				10.0	4.1	41
0.6				10.0	2.2	22
	0.25			10.0	9.5	95
	0.50			10.0	9.4	94
	0.75			10.0	8.3	83
	1.00			10.0	8.0	80
		25		10.0	9.4	94
		25		10.0	9.4	94
		25		10.0	9.5	95
			25	10.0	8.9	89
			25	10.0	8.6	86

\* The concentration of the urea and sodium chloride is calculated from the volumes of the solutions after the addition of the mercuric acetate solution.

was not removed by centrifugation until 1 hour after the addition of the mercuric acetate reagent. The remaining procedure for the experiments given in Table IV was the same as for those recorded in Table III. From an inspection of Table IV it is evident that under the conditions of these experiments, chlorides even in small amounts markedly interfere with the recovery of the allantoin. If the concentration of the urea is over 0.5 per cent, the recoveries of allantoin are also unsatisfactory. The

presence of creatine is without effect, but in the tubes containing the creatinine, the recoveries are approximately 10 per cent lower than the recoveries that are usually obtained from pure solutions of allantoin. Creatine and creatinine were included in this series, since, according to Greenwald (10), creatine is oxidized by mercuric acetate to methylguanidinoglyoxylic acid, which is hydrolyzed to methyl guanidine and oxalic acid. Apparently the conditions are unfavorable for the oxidation of the creatine and the creatinine by the mercuric acetate, since high results are not obtained when these compounds are present, as one might

TABLE V.  
*Effect of Chlorides and Urea upon the Recovery of Allantoin.\**

Allantoin present.	Urea present.	Sodium chloride present.	Allantoin recovered.	
mg.	mg.	mg.	mg.	per cent.
35.0	0	0	33.7	96
37.5	0	0	35.8	96
35.0	500	100	33.5	96
35.0	500	200	33.6	96
35.0	500	400	33.2	95
35.0	500	1000	33.7	96
35.0	750	400	31.2	89
35.0	1000	400	29.5	84
35.0	2000	1000	25.6	76

\* The amounts of allantoin, urea, and sodium chloride given in Columns 1, 2, and 3 of this table are contained in 100 cc. of solution. Each 100 cc. of solution was carried through the entire procedure proposed for the analysis of allantoin in urine.

expect if the methylguanidinoglyoxylic acid had been formed. The presence of creatinine leads to low recoveries of allantoin, but since in the method for urine, creatinine is precipitated by the phosphotungstic acid, no further study has been made of the influence of creatinine. Since uric acid is likewise removed from the urine by phosphotungstic acid, the effect of its presence upon the recovery of allantoin from aqueous solutions has not been studied.

It remains to be demonstrated that pure solutions of allantoin and mixtures of allantoin with other urinary constituents can be treated successively with each reagent, which is used in the method

previously described for urine, without the loss of significant amounts of allantoin. Table V is a record of a number of experiments of this nature. 100 cc. portions of solutions containing either pure allantoin or allantoin with varying amounts of sodium chloride and urea were treated with 5 cc. of 4 N sulfuric acid and 5 cc. of glacial acetic acid. Then 1 gm. of phosphotungstic acid was added and put into solution with shaking. Since none of the compounds in these solutions (allantoin, urea, or sodium chloride) are precipitated by the phosphotungstic acid, there is an excess of 1 gm., which must be removed as the lead salt. The solution was now treated as described in the method for the estimation of allantoin in urine. It is important to note that the allantoin is precipitated by mercuric acetate from a solution which is faintly alkaline (pH 7.2 to 7.4) due to the presence of basic lead acetate. Apparently under these conditions, sodium chloride in large amounts does not interfere with the precipitation of allantoin by mercuric acetate. The concentration of urea must not greatly exceed 0.5 per cent if good recoveries of allantoin are to be obtained.

Mixtures of allantoin and creatine, allantoin and hippuric acid, and allantoin and uric acid have been studied in a manner similar to that just described. Recoveries of allantoin ranging from 94 to 98 per cent were obtained. In no case were the recoveries over 100 per cent. As a final test of the method, two solutions containing allantoin and many of the common urinary constituents were prepared. Solution 1 contained per 100 cc.:

25 mg. allantoin.	100 mg. creatine.
500 " urea.	100 " creatinine.
300 " glucose.	200 " calcium chloride.
50 " ammonium sulfate.	25 " uric acid.
50 " sodium acid phosphate.	50 " hippuric acid.

Solution 2 contained per 100 cc. the same amount of allantoin but 50 per cent more of each of the other constituents. The analysis of these solutions for the content of allantoin was made according to the procedures previously outlined for urines. 94 per cent of the allantoin was recovered from Solution 1, and 87 per cent from Solution 2. The lower recovery of allantoin from Solution 2 may be in part due to the high content of urea, which prevents the quantitative precipitation of the allantoin.

## DISCUSSION.

Although the present method was designed primarily for the estimation of allantoin in rabbit urine, the writer knows of no reason why it cannot be applied to the urine of other animals which excrete allantoin as the principal end-product of purine metabolism. From the results obtained with solutions containing known amounts of allantoin and from the analysis of urines before and after the addition of allantoin, it is believed that the method is more accurate than those which have been used prior to this time. This is particularly true of urines which contain small amounts of allantoin.

If it is advisable to use less than 100 cc. of urine for the analysis, the amount of acid mixture must be reduced in proportion. It is not necessary that the hydrolysis of allantoin to oxalic acid be made in a volume of 25 cc., and if one is dealing with a small amount of allantoin it is better to make the hydrolysis in a 100 cc. Erlenmeyer flask, with a final volume of 10 cc. For the removal of the silicates this volume is conveniently diluted to 25 cc., and the largest possible aliquot of this solution used for the precipitation of the oxalic acid. Again if a very dilute urine is to be analyzed it is advisable to use more than 100 cc. of urine for the analysis. The mercury allantoin precipitates from several portions of the basic lead acetate filtrate may then be combined prior to the decomposition with hydrogen sulfide, and the subsequent hydrolysis with alkali.

Although the present method requires from 6 to 7 hours for completion, approximately one-half of this time is required for the actual manipulation. This is much less time than that required for the original Wiechowski method, and somewhat less than that required for the Handovsky method. The original purpose, when the research was undertaken, was to provide a short, accurate method for the determination of allantoin. It was soon found, however, that accuracy was sacrificed for speed when an attempt was made to eliminate certain steps in the procedure, which are now employed.

## SUMMARY.

A method for the determination of allantoin in rabbit urine is described, which is based upon the hydrolysis of allantoin to oxalic acid by alkali, followed by precipitation as calcium oxalate, and estimation of the latter by the usual permanganate titration.

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# THE PHOSPHORUS CONTENT OF HUMAN MILK AND COW'S MILK.

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## INTRODUCTION.

The purpose of the work presented in this paper has been to ascertain in what forms and quantities phosphorus is ingested by the breast-fed child and the artificially fed child. This problem resolved itself into a determination of the different phosphorus compounds of human and cow's milk. Previous analyses have given widely varying results as indicated by Table I.

Bosworth and Van Slyke have estimated the different combinations of inorganic phosphorus as indicated in Table II.

## EXPERIMENTAL.

Both total phosphorus and the phosphorus in separate fractions were determined by the Gregersen (1907) modification of Neumann's method as modified for micro determinations by Iversen (1920). The method has proven capable of determining from 0.1 to 0.5 mg. of phosphorus with an error of less than 4 per cent of the amount present.

For division of the phosphorus compounds into fractions the methods employed were those devised for blood analyses by Greenwald and later elaborated by Feigl and Iversen. The fractions obtained are indicated as follows:

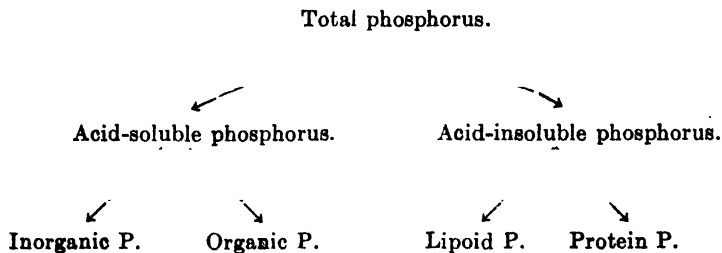


TABLE I.

Author.	Mg. P per 100 cc. of milk.
Human milk.	
Bunge, total P. ....	20.5
Pagés, " " ....	10.9
Michel (and Michel and Perret), total P. ....	19.3
Blauberg, total P. ....	12.8
Stoklasa, " " ....	19.2
Backhaus and Cronheim, total P. ....	12.1
Keller, total P. ....	17.2
Camerer and Söldner, total P. ....	12.9
de Lange, total P. ....	25.2
Schlossmann, total P. ....	19.6
Sikes, total P. ....	13.0
Schloss, total P. ....	18.1
Lindberg, total P. ....	18.4
Hamilton, total P. ....	15.0
Sikes, protein P. ....	5.6
" non-protein P. ....	7.4
Holt, Courtney, and Fales, inorganic P. ....	7.4
Von Meysenbug, inorganic P. ....	4.0
Denis (Sisson and Denis), inorganic P. ....	4.0
Cow's milk.	
Bunge, total P. ....	86.2
Schrodt and Hansen, total P. ....	About 80
Söldner, total P. ....	105.6
Bøggild, total P. ....	94.8
Stoklasa, " " ....	79.0
Blauberg, " " ....	31.6
Trunz, " " ....	About 90
Orla-Jensen, " " ....	" 90
Von Wendt, " " ....	100.7
Schepang, " " ....	76.1
Sheehy, " " ....	About 90
Duclaux, " " ....	96.1
af Klercker, " " ....	About 100

Two questions called for solution in regard to this classification:

1. Does the picric acid solution used for precipitating acid-insoluble phosphorus compounds cause any decomposition in them or in the phosphorus compounds of the filtrate?

2. Does the classification of the phosphorus compounds into acid-soluble and acid-insoluble groups have any physiological significance?

TABLE II.  
*Inorganic Phosphates of Milk.*

	Cow's milk.	Human milk.
	<i>per cent</i>	<i>per cent</i>
Dicalcium phosphate.....	0.175	0.000
Tricalcium ".....	0.000	0.000
Monomagnesium phosphate .....	0.103	0.027
Dimagnesium phosphate .....	0.000	0.000
Trimagnesium ".....	0.000	0.000
Monopotassium ".....	0.000	0.069
Dipotassium ".....	0.230	0.000

TABLE III.  
*Comparison of Acid-Insoluble P and Casein P of Cow's Milk.*

Sample No.	Acid-insoluble P.	Casein P.
	<i>mg.</i>	<i>mg.</i>
1	18.8	18.3
2	19.4	18.6
3	13.5	13.7
4	21.0	20.7
Average.....	18.2	17.8

### *Acid-Insoluble Phosphorus.*

In order to answer the first question we prepared series of mixtures of milk with equal quantities of precipitant. These mixtures were allowed to stand for periods varying from 1 to 24 hours before they were filtered and analyzed. It was found that the duration of exposure to the acid precipitant was entirely without influence on the result.

The answer to the second question is that practically all of the

acid-insoluble phosphorus is that of the casein. In four specimens of cow's milk the casein phosphorus was determined after precipitation of the casein with rennet, and the acid-insoluble phosphorus in the precipitate obtained with the usual picric acid precipitant. The results given in Table III show that the same amounts of phosphorus are precipitated by both procedures.

With *human milk* the material available permitted only a single experiment. The sample analyzed showed 11.2 mg. of acid-soluble phosphorus, 1.2 mg. of acid-insoluble phosphorus, and 1.2 mg. of casein phosphorus.

These experiments show that the casein phosphorus constitutes almost the entire fraction of acid-insoluble phosphorus in milk. There is probably also a minute amount of phosphatides, but too

TABLE IV.  
*Phosphorus in 100 Cc. of Whey.*

Sample No.	Total P.	Acid-soluble P.	Acid-insoluble P.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	48.3	47.9	0.4
2	51.2	50.3	0.9
3	55.4	53.7	1.7
4	52.2	51.5	0.7
5	53.2	52.9	0.3
Average .....	52.1	51.3	0.8

little to ascertain with certainty by the methods used. The "lipoid P" of the acid-insoluble fraction is very small.

#### *Acid-Soluble Phosphorus.*

In the acid-soluble fraction of cow's milk we have found that about five-sixths of the phosphorus is precipitable as ammonium magnesium phosphate, and may therefore be considered as inorganic. In *human milk* on the other hand only one-half the acid-soluble phosphorus is thus found to be inorganic.

#### *Phosphorus of Whey.*

The data in Table IV indicate the probability that a slight amount of phosphorus is precipitated in milk by picric acid and is

not precipitated by rennet. If this is the case we should obtain in whey a slight amount of acid-insoluble phosphorus. Table IV gives the results of analyses of five wheys and shows that in each case there is a slight amount of acid-insoluble phosphorus in excess of the casein phosphorus precipitated by rennet.

We have furthermore determined by the magnesia precipitation the inorganic and organic phosphorus fractions of whey and of the picric acid filtrate. Comparison of results obtained with the filtrates of rennet and picric acid precipitation respectively indicate that the acid precipitant was without appreciable effect in hydrolyzing organic phosphorus.

TABLE V.  
*Phosphorus in 100 Cc. of Whey.*

Milk from which whey was formed.	Total P.	Acid- insoluble P.	Acid-soluble P.			
			Total.	Inorganic.	Organic.	
					Direct.	Indirect.
	mg.	mg.	mg.	mg.	mg.	mg.
1. Cow.....	53.8	2.3	51.5	38.3	13.4	13.0
2. ".....	45.6	1.0	44.6	31.1	13.5	12.0
3. Human. ....	9.2	0.2	9.0	5.5	3.5	3.6
4. ".....	12.5	0.4	12.1	2.4	9.7	7.8
5. Cow *.....	48.3	0.1	48.2	40.8	7.4	7.5
6. " *.....	59.4	2.0	57.4	48.3	9.1	9.3
7. " *.....	55.8	0.8	55.0	46.4	8.6	8.1
8. " †.....	39.0	2.0	37.0	27.2	9.8	9.7
9. " †.....	40.4	1.3	39.1	26.3	12.8	11.7

\* Artificial whey. Filtrate after precipitation of casein with acetic acid.

† Artificial whey. Filtrate after salting out casein.

#### *Procedure for Fractionating the Phosphorus of Milk.*

As the result of the above experiments we have adopted the following procedure for determining the phosphorus fractions of milk:

1. *Total phosphorus* is determined in 0.3 to 0.5 cc. of cow's milk, or in 1 to 2 cc. of human milk, after destruction of the organic matter by the Neumann method.

2. *Acid-soluble phosphorus* is determined by precipitation of 0.5 to 1.0 cc. of cow's milk, or of 2 to 4 cc. of human milk, with 20

cc. of acid mixture. After 1 hour at room temperature the solution is filtered. The phosphorus content of 15 cc. of filtrate is determined after destruction of the organic matter.

3. *Acid insoluble phosphorus* is calculated by subtracting (2) from (1).

4. *The Organic Acid-Soluble Phosphorus*.—To 4 cc. of cow's milk or 6 cc. of human milk 20 cc. of acid precipitant solution are added. In the filtrate the inorganic phosphate is precipitated

TABLE VI.  
*Phosphorus in 100 Cc. of Human Milk.*

Analysis No.	Day of lactation.	Fat.	Total P.	Acid-insoluble P.	Acid-soluble P.		
					Total.	Inorganic.	Organic.
		<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	15	3.7	16.3	3.0	13.3	5.0	8.3
2	35	3.5	15.3	3.3	12.0	7.1	4.9
3	50	5.4	16.8	3.2	13.6	6.4	7.2
4	51	4.2	9.5	2.5	7.0	1.9	5.1
5	54	2.3	17.0	4.7	12.3	3.6	8.7
6	67	3.2	18.6	2.9	15.7	7.6	8.1
7	71	4.0	10.3	1.6	8.7	2.8	5.9
8	74	2.0	16.4	3.7	12.7	5.8	6.9
9	105	1.6	16.3	3.1	13.2	4.9	8.3
10	151	4.2	15.8	1.9	13.9	6.5	7.4
11	221	2.4	12.7	2.4	10.3	4.4	5.9
12	275	3.0	12.4	1.2	11.2	7.9	3.3
13	286	2.05	11.8	1.5	10.3	4.3	6.0
14	287	3.6	12.8	2.4	10.4	3.8	6.6
15	338	3.9	11.5	1.9	9.6	4.8	4.8
Average.....			14.2	2.6	11.6	5.1	6.5

with ammonia-magnesia mixture, and in the filtrate from this the organic acid-soluble phosphorus is determined.

5. *The inorganic phosphorus* is calculated by subtracting (4) from (2).

*Nature of the So Called Organic Acid-Soluble Phosphorus.*

Winterstein and Strickler mentioned glycerol-phosphoric acid as a normal component of colostrum. That it constitutes the organic acid-soluble phosphorus of normal human milk is uncer-

tain. We find however, that in both human milk and cow's milk a substance is present which has the following properties of glycerol-phosphoric acid. It is soluble in water and alcohol, insoluble in ether and acetone. It is not hydrolyzed by boiling in water or very dilute acetic acid. It is hydrolyzed by boiling with stronger acetic acid or with dilute mineral acids. It contains phosphorus. The phosphorus is not precipitated by ammonia-magnesia solution. When the substance is heated with sodium bisulfate it yields a small amount of acrolein.

TABLE VII.  
*Phosphorus per 100 Cc. of Cow's Milk.*

Analysis No.	Date.	Fat.	Total P.	Acid-insoluble P.	Acid-soluble P.		
					Total.	Inorganic.	Organic.
	1921	per cent	mg.	mg.	mg.	mg.	mg.
1	Nov. 2	3.2	96.0	16.0	80.0	69.5	10.5
2	" 9	3.6	89.7	14.2	75.5	64.4	11.1
3	" 16	3.8	95.8	15.8	80.0	67.8	12.2
4	" 23	3.7	96.4	17.1	79.3	67.5	11.8
5	Dec. 1	3.65	95.3	19.0	76.3	66.4	9.9
6	" 7	3.6	98.2	17.1	81.1	69.6	11.5
7	" 14	3.55	96.7	17.4	79.3	68.6	10.7
8	" 21	3.8	92.3	14.8	77.5	67.1	10.4
9	" 28	3.5	95.3	17.2	78.1	68.2	9.9
	1922						
10	Jan. 4	3.5	96.0	18.5	77.5	66.8	10.7
11	" 14	3.6	96.7	17.4	79.3	66.8	12.5
12	" 19	3.6	93.8	15.1	78.7	67.9	10.8
13	" 27	3.9	95.3	17.8	77.5	66.5	11.0
14	Feb. 2	3.5	96.7	18.6	78.1	66.1	12.0
15	" 8	3.5	98.9	22.6	76.3	64.3	12.0
Average.....			95.4	17.1	78.3	67.1	11.1

When sodium glycerophosphate is added to milk the phosphorus is exactly recovered in the acid-soluble organic phosphorus fraction.

*Phosphorus of Human Milk.*

In the manner outlined above we have determined the phosphorus fractions in fifteen samples of human milk. The results are given in Table VI.



*Phosphorus of Cow's Milk.*

For our analyses we have used a mixture of the milk from thirty selected cows found healthy by regular examinations. The milk was analyzed fresh, with precautions against contamination by bacterial decomposition or otherwise. The normality of the milk was further confirmed by determination of the fat and solid contents and specific gravity, and by microscopic examinations. The results are given in Table VII.

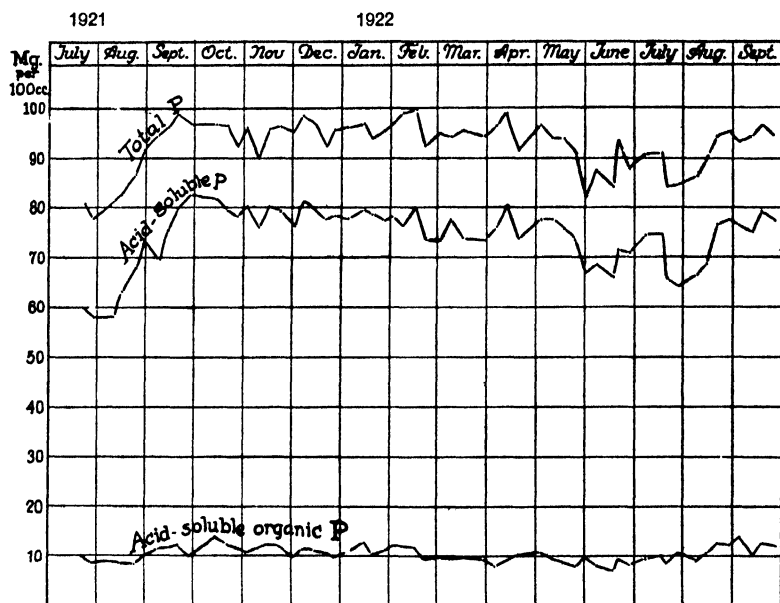


FIG. 1.

Comparison of the average results in Tables VI and VII shows that a liter of cow's milk contains 6.7 times as much total phosphorus as a liter of human milk, 6.6 times as much acid-insoluble phosphorus, 6.7 times as much acid-soluble phosphorus, 13.1 times as much inorganic phosphorus, and 1.7 times as much acid-soluble organic (possibly glycerophosphate) phosphorus.

*Seasonal Variation of the Phosphorus of Cow's Milk.*

With the idea that there might possibly be some relation between variations in the rate of incidence of rickets at different seasons

and the variations of the phosphorus fractions of milk, we have determined from week to week the different fractions in milk controlled for its normality as above described. The results are given in Fig. 1. They show that in the main the casein phosphorus and organic acid-soluble phosphorus remain the same throughout the year, but that inorganic phosphorus is high from September to May, and low during June, July, and August. The low values correspond to the period during which the animals were in pasture, the high values to the period of stall feeding.

#### SUMMARY.

Analyses of fifteen samples of normal human milk and of fifteen samples of normal herd milk of cows yielded the following as the average amounts of phosphorus in the indicated fractions:

	Mg. of phosphorus per 100 cc. of milk.				
	Total P.	Acid-insoluble P.*	Acid-soluble P.		
			Total.	Inorganic.	Organic.
Human milk .....	14.2	2.6	11.6	5.1	6.5
Cow's " .....	95.4	17.1	78.3	67.1	11.2

\* Almost entirely casein P, with a trace of lipid P.

The acid-insoluble phosphorus (precipitated by picric acid) was found to be about 98.5 per cent casein phosphorus, with a trace of lipid phosphorus.

Weekly determinations of the phosphorus fractions in herd milk showed the same values for casein and acid-soluble organic phosphorus throughout the year. Inorganic phosphorus, however, was lower during the 3 summer months, when the animals were in pasture, than during the other 9 months.

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## NOTE ON DONNAN EQUILIBRIUM AND OSMOTIC PRESSURE RELATIONSHIP BETWEEN THE CELLS AND THE SERUM.

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(Received for publication, June 25, 1926.)

In a paper on the factors controlling the electrolyte and water distribution in the blood Van Slyke, Wu, and McLean (1) based their mathematical deduction on three fundamental postulates: (1) Approximate neutrality of the blood reaction; sum of base cations equals sum of anions. (2) Validity of Donnan's law of membrane equilibrium. (3) Equality of the osmotic pressure of the fluid within the cells and that of the serum outside.

The first two postulates stood on experimental data familiar in the literature. The third postulate was without experimental basis. That it was substantially correct was shown by the osmolar concentration data presented in Table I of their paper and by the general agreement between the observed effects of changes of  $O_2$  and  $CO_2$  tensions on the water and electrolyte distribution on the one hand with those predicted by the aid of those postulates on the other hand (1, 2).

It deserves emphasis, however, that osmotic *inequality* would be the condition of a Donnan equilibrium in which ions of *only one sign were indiffusible*, and that the approximate osmotic equality between the cells and the serum is the result of a special condition obtaining in blood; namely, the *impermeability of the cell membrane to the metallic cations as well as to the protein anions*.

Let us assume, for the sake of simplicity, that the cell contained only sodium bicarbonate and oxyhemoglobin and the serum contained only sodium bicarbonate. The oxyhemoglobin is present partly as salt and partly as free protein. Then at equilibrium, if the cation  $B^+$  were diffusible, like the anion  $A^-$ ,

we would have, using the symbols of Van Slyke, Wu, and McLean (1),

$$\frac{[B^+]_c}{[B^+]_s} = \frac{[A^-]_c}{[A^-]_s} = \frac{[H^+]_c}{[H^+]_s} \quad (1)$$

$$[A^-]_c + [HbO_2^-]_c = [B^+]_c \quad (2)$$

$$\text{and } [A^-]_s = [B^+]_s \quad (3)$$

The  $[H^+]$  and  $[OH^-]$  being negligible, the difference in total ionic concentration between the cells and the serum would be

$$([B^+]_c + [A^-]_c + [HbO_2^-]_c) - ([B^+]_s + [A^-]_s) = 2([B^+]_c - [B^+]_s).$$

Since  $[H^+]_c$  is greater than  $[H^+]_s$ ,  $[B^+]_c$  would also be greater than  $[B^+]_s$ .

Calculation shows that the osmotic pressure difference corresponding to  $([B^+]_c + [A^-]_c) - ([B^+]_s + [A^-]_s)$  would cause such a swelling of the cells that the membranes would burst. That this does not occur is an independent proof of the *indiffusibility of the cations*.

As the pH of the blood is lowered towards the isoelectric point of the oxyhemoglobin by addition of a diffusible monovalent acid, HA, part of the hemoglobin in the form of the polyvalent salt  $B_n(HbO_2)$  loses its base to the invading acid. This results in the replacement of  $B_n(HbO_2)$ , of which only the  $B^+$  ions exert significant osmotic pressure, by the salt BA, of which both  $B^+$  and  $A^-$  are osmotically active. There is consequently an increase in the molal concentration in the cells. If we assume that the oxyhemoglobin molecule has been combined with 3 equivalents of monovalent base, and is displaced from that combination by carbonic acid, then in place of 1 oxyhemoglobin anion, we have 1 oxyhemoglobin molecule and 3  $HCO_3^-$  ions. The oxyhemoglobin molecule has presumably the same osmotic activity as the oxyhemoglobin anion. The 3  $HCO_3^-$  anions formed therefore represent a net gain of three osmotically active particles to the cell fluid. There being no increase of molal concentration in the serum, a shift of water from the serum to the cells takes place. Such a shift was observed by Van Slyke, Wu, and McLean (1) and approximated in extent that calculated on the above assumption.

With fall of pH towards the isoelectric point of the protein-con-

taining phase of a two-phase system in Donnan equilibrium, Loeb's (3) experiments with gelatin showed that the direction of water transfer is from the protein-containing phase to the protein-free one. Base cations held by the gelatin became diffusible by combining with the diffusible acid which had lowered the pH, and with the anions of the acid passed out into the protein-free phase taking water with them. But in the blood the transfer of water is in the *opposite direction*: adding acid causes the cells to swell. The reason for the difference again is that the base in the blood cells cannot diffuse out of them. The potassium cations of the cells do not become diffusible by combining with diffusible anions. On the contrary they anchor such anions within the cells, and consequently draw water in with them.

We may therefore distinguish between two kinds of Donnan equilibria, one in which only the "colloidal" ions of relatively low osmotic activity are indiffusible, and another in which also osmotically active "crystalloid" ions, of the charge opposite to that of the colloid, are indiffusible. In equilibria of the former kind equality of osmotic activity in the two phases is impossible (except at the isoelectric point of a colloid with molecules of zero osmotic activity). In equilibria of the latter kind, of which the blood is an example, the concentrations of ionic charges in the two phases are variable, but the total sum of charges in each is constant, the variability in concentration being caused by water transfer. In a system of this kind, as charges shift from osmotically inactive colloids to osmotically active crystalloid ions, water can pass in either direction unaccompanied by electrolyte in such a manner as to maintain osmotic equality in the two phases.

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# THE COLORIMETRIC ESTIMATION OF CHOLESTEROL AND LECITHIN IN BLOOD IN CONNECTION WITH FOLIN AND WU'S SYSTEM OF BLOOD ANALYSIS.

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(Received for publication, June 9, 1926.)

## *Principle.*

The protein precipitate obtained by Folin and Wu's system is washed, dried, and extracted with hot chloroform in a similar manner to that employed by Myers and Wardell (1) for total blood. In this chloroform extract cholesterol is estimated as usual by means of Liebermann's reaction, and lecithin is determined as lipid phosphorus by Whitehorn's (2) recent method.

## *Technique.*

The requisite quantity of blood (5 to 10 cc.) is dealbuminated by the technique of Folin and Wu. The filtrate must be passed through a filter which has been thoroughly freed from all fat. (The filtrate may be kept for the determination of urea, sugar, etc.) The flask in which the precipitation has taken place must then be rinsed out several times with distilled water, and the water thrown onto the protein precipitate. While still wet, precipitate and filter paper are placed in a small mortar and mixed carefully with 8 to 10 gm. of plaster of Paris or sea sand, previously cleansed thoroughly of all fat. Thus a homogeneous paste is obtained, more or less thick, according to the quantity of sea sand added.

The paste is placed for  $1\frac{1}{2}$  to 2 hours in an oven at 100–105°C. Afterwards triturate it carefully and transfer it to a thimble for extraction, stopping up the free aperture with cotton free of fat. Use the Kumagawa-Suto (3) apparatus for the extraction and employ redistilled chloroform. The extraction should take at



least 2 hours; care must be taken to see that the syphon is in perfect working order, otherwise prolong the period of extraction. When the latter is finished, the surplus chloroform must be distilled until only a residue of 10 to 15 cc. remains. This is transferred to a 50 or 100 cc. volumetric flask, according to the amount (5 or 10 cc.) of blood used for the precipitation. The extraction flask is then washed out with 10 cc. of chloroform three separate times, the washings being transferred to the volumetric flask. The solution in the flask is finally brought up to volume.

#### *Determination of Cholesterol.*

Exactly 10 cc. of this chloroform extract are measured out into a small flask; 4 cc. of acetic anhydride and 0.2 cc. of concentrated sulfuric acid are added. This is then put into a dark place for half an hour at a temperature of from 20–30°C. The color is compared with that of a standard solution composed as follows: 0.15 gm. of purest cholesterol (Merck) is dissolved in 100 cc. of redistilled chloroform and put into small bottles containing about 15 cc. each. These are closed with glass stoppers, sealed with paraffin, and kept in an ice box. By taking 10 cc. of this original solution and bringing to 100 cc. with redistilled chloroform the standard solution is obtained: 10 cc. of the latter contain therefore exactly 1.5 mg. of cholesterol.

The calculation is simple, since 10 cc. of chloroform extract equal 1 cc. of total blood.

#### *Determination of Lecithin.*

The amount of lecithin in another portion of this chloroform extract can be determined by employing one of the micro methods recommended for this purpose. I have preferred using Whitehorn's phosphorus method, taking 5 cc. of the extract.

In order to determine whether the deproteinization recommended by Folin and Wu would have any adverse effect on the colorimetric reactions used in the determination of cholesterol and of lipid phosphorus I made some tests; Tables I and II serve as examples of the results.

The gravimetric determination advocated by Windaus (4) was employed on the petroleum ether extract obtained by treating

20 cc. of blood by the Kumagawa-Suto method, modified by Shimidzu (5).

Kumagawa-Suto-Shimidzu's method of colorimetric determination was employed, always on petroleum ether extract, soluted in

TABLE I.  
*Comparative Determination of Cholesterol in Total Ox Blood.*

Method.	Cholesterol.
	<i>mg. per cent</i>
Kumagawa-Suto-Shimidzu-Windaus (gravimetric) .....	128
Kumagawa-Suto-Shimidzu (colorimetric) .....	136
Myers-Wardell, 1st sample .....	125
2nd " .....	138
Grigaut, 1st sample .....	139
2nd " .....	146
Autenrieth-Lichtenthaeler, 1st sample .....	114
2nd " .....	122
Original method, 1st sample .....	132
2nd " .....	140

TABLE II.  
*Comparative Determination of Lipoid Phosphorus in Total Ox Blood.*

Method.	Lipoid phosphorus.	Lecithin (calculated).
	<i>mg. per cent</i>	<i>mg. per cent (× 25.6)</i>
Whitehorn, 1st sample .....	9.3	238
2nd " .....	9.9	253
Original method, 1st sample .....	8.7	223
2nd " .....	9.6	246

chloroform, etc., in accordance with the instructions contained in Abderhalden's book (6).

I must again lay stress on the fact that both in the Myers-Wardell method and in mine, great care must be taken to ensure

the good working of the syphon, otherwise the extraction may be incomplete and should be prolonged.

An examination of these tables shows that the results contain the usual percentage of error in connection with the methods used as a basis for the method I have proposed. Further technical improvements will doubtless yield more accurate results. I may, however, conclude for the present that deproteinization according to Folin and Wu's method in no way prevents the determination of cholesterol and lecithin on the protein precipitate which, instead of being thrown away as at present, can be used for this purpose with advantage.

#### SUMMARY.

A method is described by which blood cholesterol and lecithin are determined by analysis of the protein precipitate of Folin and Wu.

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## THE CONFIGURATIONAL RELATIONSHIPS OF DIALKYLACETIC ACIDS.

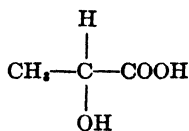
BY P. A. LEVENE AND LAWRENCE W. BASS.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

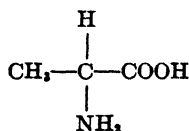
(Received for publication, July 20, 1926.)

The present paper is a report on the beginning of an investigation of the configurational relationships of dialkylacetic acids and of substances derived from them, the latter differing from one another in the character of the active group.

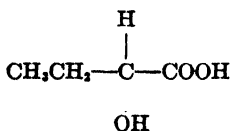
In the series of hydroxy and of amino acids the configurationally related substances often rotate in opposite directions. Thus, levo-lactic, dextro-2-hydroxybutyric, levo-2-hydroxyisovaleric, and dextro-2-hydroxyisohexoic acids are configurationally related; similarly, dextro-alanine, dextro-valine, levo-leucine, and dextro-isoleucine are configurationally related. Referring to these substances as substituted acetic acids, the similarity in structure is apparent.



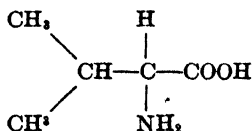
Levo.



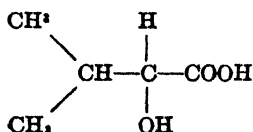
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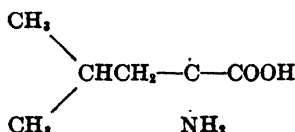
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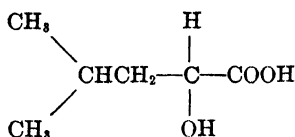
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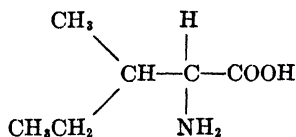
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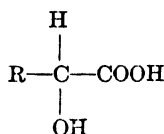


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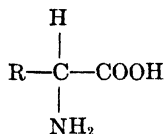


Dextro.

The substances in the first column may be represented by the general formula

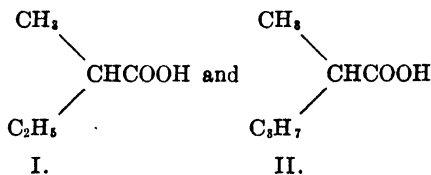


those in the second column by



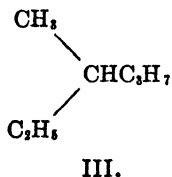
Thus a change in the radicle R often leads to a change in direction of rotation in configurationally related substances.

The question then arises: Do similar relationships exist when the two substituting groups in acetic acid are alkyl radicles? In other words, do

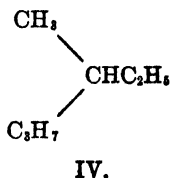


rotate in the same direction when they are configurationally related or when they are enantiomorphous? The problem can be solved in the following way. Assuming that the substances I and II are configurationally related when they rotate in the same direction,

let us convert I into



and II into



These two hydrocarbons should then be stereoisomers; *i.e.*, they should rotate in opposite directions. On the other hand, if they (III and IV) rotate in the same direction, the observation would lead to the conclusion that dextro-ethylmethylacetic acid and dextro-propylmethylacetic acid are enantiomorphous.

Fortunately, some data, although very scanty, needed for the solution of the problem, are already recorded in the literature. The observations are limited to derivatives of the naturally occurring active amyl alcohol. Marckwald,<sup>1</sup> on one hand, oxidized the levo-alcohol to dextro-ethylmethylacetic acid and, on the other hand, converted it into dextro-amyl halides, into dextro-amylamine, and into dextro-methylethylpropylmethane. It is thus only necessary to convert active methylpropylacetic acid into methylethylpropylmethane in order to correlate these two substituted acetic acids. The higher members of the series will then be correlated in a similar manner.

Another object of the present investigation is to follow the influence of the change in the polarity of the group substituted for the carboxyl on the direction of the rotation of the resulting substance. Thus, the radicle  $-\text{COOH}$  may be readily reduced to  $-\text{CH}_2\text{OH}$  and in this compound the hydroxyl group may be replaced by any

<sup>1</sup> Marckwald, W., *Ber. chem. Ges.*, 1904, xxxvii, 1038.

desired polar group. In fact, some of these transformations constitute intermediate steps in the solution of the first problem.

The work thus far has progressed only to the stage of the synthesis and resolution of some of the substituted acetic acids required for the solution of our problem. Those which have been resolved are 2-*n*-propyl-, 2-isopropyl-, 2-*n*-butyl-, and 2-isobutylpropionic acids. The points of interest in connection with the rotations of these four acids are, first, that substitution of a branched chain radicle for a normal chain apparently has little influence on the numerical value of the molecular rotation of the resulting substance, and, second, that the increase in the molecular weight of the radicle from propyl to butyl causes a marked increase in the molecular rotation. These conclusions are made evident in Table I.

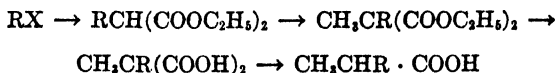
TABLE I.

	$[\alpha]_D^{25}$	$[\text{M}]_D^{25}$
2- <i>n</i> -Propylpropionic acid.....	-13.2°	-15.3°
2-Isopropylpropionic acid.....	-13.9°	-16.1°
2- <i>n</i> -Butylpropionic acid.....	+19.6°	+25.5°
2-Isobutylpropionic acid.....	+19.4°	+25.2°

## EXPERIMENTAL.

*I. Preparation of 2-Alkylpropionic Acids.*

The preparation of the 2-alkylpropionic acids employed in this investigation (2-ethyl-, 2-*n*-propyl-, 2-isopropyl-, 2-*n*-butyl-, and 2-isobutylpropionic acids) was accomplished by means of the malonic ester synthesis, which may be represented schematically as follows:



Since the preparations of all the acids which we have synthesized are described in the literature, the report on this phase of the work is limited to a brief summary of the standardized procedure which we have adopted.

In the synthesis of dialkylmalonic esters, the heavier alkyl

group should be introduced first. This fact, which is stated in the literature, has been confirmed by our experience. Furthermore, the importance of drying very carefully all the products used in the condensations should be emphasized.

Particular attention was paid to the purification of the various intermediate products, which were all analyzed.

*Preparation of Alkylmalonic Esters.*—The procedure used in the first step of the synthesis was essentially a small scale adaptation of the method described by Adams and Kamm<sup>2</sup> for the preparation of *n*-butylmalonic ester. The reactions were carried out in molar quantities with yields of 75 to 90 per cent.

In the preparation of the propyl and butyl derivatives the use of the alkyl iodides instead of the alkyl bromides showed no marked advantages. The iodide was used exclusively in the case of the ethyl derivative.

*Preparation of Methylalkylmalonic Esters.*—The preparation of these esters from the purified products obtained in the preceding step was carried out by an analogous procedure in  $\frac{1}{2}$  molar quantities with yields of 70 to 85 per cent. The purification of the esters by fractional distillation presents some difficulties because of the proximity of the boiling points of the alkylmalonic esters and their respective methyl derivatives.

*Preparation of Methylalkylmalonic Acids.*—Molar quantities of the methylalkylmalonic esters from the preceding preparation were saponified by refluxing for 10 hours with 3 mols of KOH in 80 per cent alcohol. The reaction mixtures were evaporated nearly to dryness and were then taken up in water. The free acids were recovered from these solutions, after acidification with 25 per cent H<sub>2</sub>SO<sub>4</sub>, by thorough extraction with ether, and subsequent evaporation of the ether. The crude acids, which were obtained with yields of 85–90 per cent, were purified by recrystallization.

*Preparation of Methylalkylacetic Acids (2-Alkylpropionic Acids).*—The purified malonic acids were decarboxylated in 150 gm. lots in 250 cc. double neck distilling flasks heated in a metal bath at 170–190°, the temperature being regulated below the boiling points of the respective alkylpropionic acids. Upon cessation of the evolution of carbon dioxide, the temperature of the metal bath was dropped to 125–145° C., and the acids were distilled under diminished pressure, a 2 or 3° fraction being taken. The yields were above 90 per cent.

## II. Resolution of 2-Alkylpropionic Acids.

2-*n*-Propyl-, 2-isopropyl-, and 2-*n*-butylpropionic acids were resolved by fractional crystallization of their quinine salts from acetone. 2-Isobutylpropionic acid was resolved by fractional crystallization of its brucine salt from acetone.

<sup>2</sup> Adams, R., and Kamm, R. M., *Organic syntheses*, New York, 1925. iv, 11.



The brucine salt of 2-ethylpropionic acid was resolved by Schütz and Marckwald<sup>3</sup> by fractional crystallization from water. Our attempt to resolve the salt by crystallization from acetone was unsuccessful because of the separation of the alkaloid instead of the salt. A partial resolution was effected, however, the alkaloid salt of the levorotatory acid being the more insoluble.

It is interesting to note that in the resolutions by means of quinine, the more insoluble salt is that of the levorotatory acid in the case of the two propyl derivatives, of the dextrorotatory acid in the case of the *n*-butyl derivative. Similarly, with brucine, the more insoluble salts are those of the levorotatory ethyl derivative and of the dextrorotatory isobutyl derivative.

The resolutions were followed by decomposing 3.0 gm. samples of the alkaloid salts with 15 cc. of 10 per cent  $\text{H}_2\text{SO}_4$  and extracting the free organic acid with ether. The ethereal extracts, after careful washing, first with dilute  $\text{H}_2\text{SO}_4$  and then with water, were dried over sodium sulfate and the ether was removed by distillation under diminished pressure. The residues of acid were used for taking the rotations, all of which were made in 3 to 6 per cent ethereal solution.

It was assumed that maximum resolution had been attained when the specific rotation remained constant within the limits of error after three successive recrystallizations of the salt.

*Resolution of 2-n-Propylpropionic Acid.*—To a hot solution of 70 gm. of the acid in 500 cc. of acetone was added one equivalent (228 gm.) of quinine and the solution was then cooled to crystallization. A duplicate experiment was begun and the two experiments were carried separately through four fractional crystallizations from acetone. The two series of crystallizations were then combined and four additional fractionations were carried out. An ethereal solution of the free acid obtained by the procedure described above from the last crop of crystals had a rotation of  $[\alpha]_D^{25} = -13.1^\circ$  (No. 416). After three more fractionations the value was

No. 434. 
$$[\alpha]_D^{25} = \frac{-0.65^\circ \times 100}{1 \times 4.92} = -13.2^\circ.$$

*Resolution of 2-Isopropylpropionic Acid.*—To a hot solution of 90 gm. of the acid in 500 cc. of acetone was added one equivalent

<sup>3</sup> Schütz, O., and Marckwald, W., *Ber. chem. Ges.*, 1896, xxix, 52.

(293 gm.) of quinine and the solution was then cooled to crystallization. A duplicate experiment was begun and the two experiments were carried separately through four fractional crystallizations from acetone. The two series of crystallizations were then combined and three additional fractionations were carried out. An ethereal solution of the free acid obtained from the last crop of crystals had a rotation of  $[\alpha]_D^{22} = -13.7^\circ$  (No. 99). After three more recrystallizations the value was

$$\text{No. 122.} \quad [\alpha]_D^{22} = \frac{-0.51^\circ \times 100}{1 \times 3.67} = -13.9^\circ.$$

*Resolution of 2-n-Butylpropionic Acid.*—To a hot solution of 65 gm. of the acid in 500 cc. of acetone was added one equivalent (189 gm.) of quinine and the solution was then cooled to crystallization. The salt was subjected to five recrystallizations from acetone. An ethereal solution of the free acid obtained from the last crop of crystals had a rotation of  $[\alpha]_D^{22} = +19.7^\circ$  (No. 228). After three more recrystallizations the value was

$$\text{No. 244.} \quad [\alpha]_D^{22} = \frac{+1.08^\circ \times 100}{1 \times 5.50} = +19.6^\circ.$$

*Resolution of 2-Isobutylpropionic Acid.*—To a hot solution of 100 gm. of the acid in 500 cc. of acetone was added one equivalent (360 gm.) of brucine and the solution was then cooled to crystallization. The salt was subjected to eight recrystallizations from acetone. An ethereal solution of the free acid obtained from the last crop of crystals had a rotation of  $[\alpha]_D^{22} = +19.1^\circ$  (No. 423). After three more recrystallizations the value was

$$\text{No. 438.} \quad [\alpha]_D^{22} = \frac{+1.01^\circ \times 100}{1 \times 5.23} = +19.4^\circ.$$



## STUDIES ON RACEMIZATION.

### IV. ACTION OF ALKALI ON KETOPIPERAZINES AND ON PEPTIDES.

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In previous communications of this series, results were communicated on the action of alkali on ketopiperazines and on di-, tri-, and tetrapeptides. The experiments have now been extended to a new tetrapeptide, namely, to glycyl-levo-alanyl-levo-alanylglycine. New observations have also been made on levo-alanylglycine anhydride. All the results thus far obtained, the previous and the new, are summarized in Table I.

A scrutiny of this table shows that the differences in the rate of racemization in ketopiperazines and in amino acids are very striking.

1. Taking intervals not exceeding 24 or 48 hours and low concentrations of alkali (about 0.1 normal), one observes about 70 to 80 per cent of racemization in the ketopiperazines, whereas the racemization of the peptides is practically nil.

2. Increasing the concentration of alkali to 1.0 normal (about 10 equivalents) and keeping the interval unchanged as in (1), it is found that the racemization in both groups of substances is minimal and for practical purposes may be regarded as negligible. The reason for the lack of racemization in ketopiperazines is explained by the fact that with the increase in concentration of alkali, the rate of hydrolysis of the ketopiperazine into the dipeptide is increased so that the former exists in solution as such not longer than 90 to 100 minutes.

3. Following the rates of racemization of peptides it is noted that unlike the case of ketopiperazines, the racemization is increased with the increase in concentration of alkali and with the increase in the time of action. The increase in temperature also has an accelerating effect on racemization.

TABLE I.

Substance.	Concen- tration.	Concentration of NaOH.		Tem- per- ature.	Duration.	Racemiza- tion.
		Nor- mality.	Equiva- lents.			
	<i>mols</i>			<i>°C.</i>		<i>per cent</i>
Dextro-alanyl-dextro-alanine anhydride.	0.07	1.0	1	3	From 24 to 72 hrs.	0
	0.10	0.1	1	20	24 "	72
Levo-alanyl-glycine anhydride.	1 0.24	0.05	0.25	15	48 "	13
	2 0.16	0.1	0.7	15	48 "	46
	3 0.16	0.1	0.7	18	48 "	67
	4 0.16	1.0	7	15	48 "	7
	5 0.24	1.0	5	18	48 "	11
	6 0.16	0.5	3.5	25-28	15 days.	20
Levo-prolyl-glycine anhydride.	0.14	0.1	0.7	18	24 hrs.	80
Levo-alanyl-glycine.	0.10	0.1	1	20	48 "	6
	0.10	1.0	10	20	48 "	0
Glycyl-levo-alanyl-glycine.	0.20	1.0	5	20	48 "	5
	0.20	0.1	0.5	20	48 "	7
	0.17	0.5	3	20	50 days.	10
Glycyl-glycyl-levo-alanyl-glycine.	1 0.11	0.1	0.9	20	48 hrs.	0
	2 0.11	0.2	1.8	20	48 "	0
	3 0.11	1.0	9	20	48 "	0
	4 0.11	0.2	1.8	20	39 days.	0
	5 0.11	0.5	4.5	20	18 "	7
	6 0.11	0.5	4.5	20	39 "	13
Glycyl-levo-alanyl-levo-alanyl-glycine.	1 0.10	0.1	1	22	4 "	0
	2 0.10	0.2	2	22	4 "	19
	3 0.10	0.5	5	22	4 "	33
	4 0.10	0.1	1	25	5 "	7
	5 0.10	0.2	2	25	5 "	20
	6 0.10	0.5	5	25	5 "	36
	7 0.10	0.1	1	22.5	9 "	8
	8 0.10	0.2	2	22.5	9 "	29
	9 0.10	0.5	5	22.5	9 "	40
	10 0.10	0.5	5	12.5	12 "	14
	11 0.10	0.5	5	12.5	28 "	27
	12 0.10	0.5	5	25	12 "	36

4. Comparing the rates of racemization of individual peptides it is observed that they rise with the increase in the number of amino acids entering into the structure of the peptide. This is easily explained by the fact that at a given interval and given concentration of alkali, the ratio of  $\frac{\text{amino acids}}{\text{peptides}}$  falls with the rise in the number of amino acids in the peptide. Of special theoretical interest is the observation that dipeptides also are capable of racemization. Thus, in Observation 7 on levo-alanyl glycine anhydride, the racemization cannot be attributed to the ring structure inasmuch as after 24 hours the ring structure does not persist. Dakin has postulated a theory that a system of three amino acids is required to make racemization possible. Dakin's theory will have to be modified if the observation on dipeptides is substantiated by further experiments. It may be added that even if racemization of dipeptides should be possible, the view of Dakin may still hold in a modified way; namely, his system may be found to be not an absolutely necessary condition, but one in which racemization proceeds more favorably.

5. Comparing the rates of racemization of the two tetrapeptides it is noted that the rate of racemization of glycyl-levo-alanyl-levo-alanyl-glycine is higher than that of diglycyl-levo-alanyl-glycine. It remains to be decided whether this difference is due to higher resistance of the former, or to structural differences.

6. Alanine itself exposed to the action of 0.1, 0.2, and 1.0 normal alkali for a period of 35 days remained unchanged in its optical activity.

The work is being extended to a larger number of ketopiperazines and peptides and also to proteins. A few preliminary experiments have already been made on gelatin and apparently no racemization was detected after short intervals. These results will be communicated later.

#### EXPERIMENTAL.

*Preparation of Levo-Bromopropionyl-Levo-Alanyl-Glycine.*—12 gm. of levo-alanyl-glycine were dissolved in 41.2 cc. of 2 N sodium hydroxide (1 equivalent) and treated alternately with 17.6 gm. of levo-bromopropionyl chloride (1.25 equivalents) dissolved in 50 cc. of ether, and 61.8 cc. of 2 N sodium hydroxide under cooling, each reagent being added in ten equal portions. The solution was then neutralized with 18 cc. of 5 N

hydrochloric acid. On cooling overnight, a precipitate of 8.5 gm. of pure levo-bromopropionyl-glycine was obtained. A second crop of 4 gm. was obtained by concentrating the filtrate to about 30 cc. and cooling as before. This second crop may be purified by recrystallizing from a small amount of hot water, or from a mixture of acetone and petroleic ether. The substance is very soluble in hot water and absolute alcohol, slightly soluble in acetone and ethyl acetate, and insoluble in ether and petroleic ether. It melted at 151–154°C. For analysis it was dried under reduced pressure over sulfuric acid at 100°C.

0.0994 gm. substance required (Kjeldahl) 7.00 cc. 0.1 N HCl.

$C_8H_{13}O_4N_2Br$ . Calculated. N 9.96.

Found. " 9.85.

It had an optical rotation of:

$$[\alpha]_D^{20} = \frac{+0.78^\circ \times 100}{1 \times 2.977} = +26.2^\circ.$$

In another preparation an optical rotation of +28° was obtained.

*Preparation of Levo-Alanyl-Levo-Alanyl-Glycine.*—15 gm. of levo-bromopropionyl-alanyl-glycine were allowed to stand 3 days at room temperature with 75 cc. of ammonium hydroxide (sp. gr. 0.90). The solution was then concentrated under reduced pressure to a thick syrup. The syrup was heated on the water bath with about 150 cc. of absolute alcohol until crystallization took place. The yield was 10.5 gm. The material was purified by dissolving it in 40 cc. of hot water and adding five parts of hot absolute alcohol. The yield of recrystallized material was 7.7 gm. It melted at 241–242°C. with decomposition. For analysis it was dried at 100°C. over sulfuric acid under reduced pressure.

0.1000 gm substance required (Kjeldahl) 13.73 cc. 0.1 N HCl.

$C_8H_{13}O_4N_2$ . Calculated. N 19.35.

Found. " 19.22.

It had an optical rotation of:

$$[\alpha]_D^{20} = \frac{+1.37^\circ \times 100}{1 \times 2.97} = +46.1^\circ.$$

In another preparation a value of +47.2° was obtained.

*Preparation of Chloracetyl-Levo-Alanyl-Levo-Alanyl-Glycine.*—8.6 gm. of levo-alanyl-levo-alanyl-glycine ( $\frac{1}{2}$  mol) were dissolved in 40 cc. of N sodium hydroxide and treated alternately with 5.4 gm. of chloracetyl chloride (1.2 equivalents) dissolved in 50 cc. of ether, and 80 cc. of N sodium hydroxide under cooling, each reagent being added in ten equal portions. The solution was neutralized with 14.4 cc. of 5 N hydrochloric acid and evaporated to dryness under reduced pressure. To remove the last traces of moisture, a little absolute alcohol was added to the residue, and the contents of the flask again evaporated to dryness under reduced pressure. The residue was extracted several times with hot acetone (total 150 cc.) and the combined acetone extracts were treated with an equal quantity of

chloroform. On cooling overnight a precipitate of 9 gm. of chloracetyl-levo-alanyl-levo-alanyl-glycine was obtained. Another  $\frac{1}{2}$  gm. may be obtained by treating the filtrate with an excess of ether. The substance is very soluble in water, hot absolute alcohol, and hot acetone, less soluble in hot ethyl acetate, and insoluble in chloroform, benzene, ether, and petroleic ether. It melted at 180°C. For analysis, it was dried over sulfuric acid under reduced pressure.

0.1000 gm. substance required (Kjeldahl) 10.20 cc. 0.1 N HCl.

$C_{10}H_{16}O_6N_4Cl$ . Calculated. N 14.31.

Found. " 14.28.

It had an optical rotation of:

$$[\alpha]_D^{20} = \frac{+2.85^\circ \times 100}{1 \times 3} = +95^\circ.$$

*Preparation of Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine.*—10 gm. of chloracetyl-levo-alanyl-levo-alanyl-glycine were allowed to stand 24 hours at room temperature with 50 cc. of ammonium hydroxide (sp. gr. 0.90). The solution was then concentrated under reduced pressure to a thick syrup. The syrup was then taken up in about 5 cc. of hot water and poured into 200 cc. of hot absolute alcohol. Crystallization began at once and was completed by heating on the water bath. The material was purified by dissolving it in 25 cc. of hot water and adding five parts of hot absolute alcohol. The yield of recrystallized material was 6.8 gm. It melted with decomposition at 263–265°C. For analysis it was dried under reduced pressure over sulfuric acid at 130°C.

0.0991 gm. substance: (Dumas) 17.10 cc.  $N_2$  at 756.7 mm. and 23°C.

0.1014 " " : 0.6320 gm.  $CO_2$  and 0.0616 gm.  $H_2O$ .

$C_{10}H_{16}O_6N_4$ . Calculated. C 43.80, H 6.57, N 20.44.

Found. " 43.89, " 6.79, " 20.10.

It had an optical rotation of:

$$[\alpha]_D^{20} = \frac{+3.03^\circ \times 100}{1 \times 2.892} = +104.8^\circ.$$

### *Experiments with Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine.*

The general procedure was the same as that described in previous publications.

*Experiment 1.*—0.5480 gm. of the peptide was dissolved in 20 cc. of 0.1 N alkali. The solution was allowed to stand 4 days at 22°C. At the end of the experiment 10 cc. of the solution of the hydrolyzed material contained in 10 per cent hydrochloric acid, 0.0666 gm. of total nitrogen (amino nitrogen was practically the same). The rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.64^\circ \times 100}{2 \times 2.12} = -15.0^\circ.$$



*Experiment 2.*—0.5480 gm. of the substance was dissolved in 20 cc. of 0.2 N alkali and the solution was allowed to stand 4 days at 22°C. At the end of the experiment 10 cc. of the solution of hydrolyzed material contained 0.0563 gm. of nitrogen. The rotation of the alanine was:

$$\frac{-0.47^{\circ} \times 100}{2 \times 1.79} = -13.1^{\circ}$$

*Experiment 3.*—0.5480 gm. of the substance was dissolved in 20 cc. of 0.5 N alkali. The duration of the experiment was as in Experiments 1 and 2. Total nitrogen in 10 cc. of the hydrolyzed material at the end of the experiment was 0.0563 gm. The rotation of the alanine was:

$$[\alpha]_t^{\circ} = \frac{-0.37^{\circ} \times 100}{2 \times 1.83} = -10.1^{\circ}$$

In this set of experiments, the change in rotation of the original substance with time was measured in order to form some idea as to the extent of hydrolysis in each experiment. Also the ratios of  $\frac{\text{amino N}}{\text{total N}}$  were determined.

	0 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20*</sup>	2 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20</sup>	4 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20</sup>	24 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20</sup>	4 days [ $\alpha$ ] <sub>D</sub> <sup>20</sup>	Ratio of $\frac{\text{amino N}}{\text{total N}}$
Concentration of alkali: 0.1 N	+4.98	+5.05	+5.03	+5.02	+5.10	$\frac{30.5}{100}$
“ “ “ 0.2 “	+5.04	+5.00	+5.00	+4.71	+4.28	$\frac{31.7}{100}$
“ “ “ 0.5 “	+4.84	+4.75	+4.58	+3.50	+2.31	$\frac{44.0}{100}$

\* l = 200 mm.

Thus, it is seen that with increase in concentration of alkali, the rate of hydrolysis increases. Notwithstanding this, the rate of racemization is also increased with the increase in concentration of alkali.

*Experiment 4.*—The same as Experiment 1; the duration of the

experiment was 6 days. At the end of the experiment 10 cc. of the hydrolyzed material contained in 10 per cent hydrochloric acid 0.0696 gm. of nitrogen. Hence, the rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.63^\circ \times 100}{2 \times 2.21} = -14.2^\circ.$$

*Experiment 5.*—The same as Experiment 2; the duration of the experiment was 6 days. 10 cc. of the hydrolyzed substance contained 0.0728 gm. of nitrogen. Hence, the rotation of the alanine was:

$$\frac{-0.56^\circ \times 100}{2 \times 2.32} = -12.1^\circ.$$

*Experiment 6.*—The same as Experiment 3; the duration of the experiment was 6 days. At the end of the experiment, 10 cc. of the hydrolyzed material contained 0.0714 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.42^\circ \times 100}{2 \times 2.27} = -9.25^\circ.$$

Also in these three experiments the changes in rotations with respect to time were measured and the ratios of  $\frac{\text{amino N}}{\text{total N}}$  were determined.

	0 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20-22*</sup>	19 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20-22</sup>	30 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20-22</sup>	54 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20-22</sup>	78 hrs. [ $\alpha$ ] <sub>D</sub> <sup>20-22</sup>	Ratio of amino N total N.
Concentration of alkali: 0.1 N.	+4.92	+5.03	+5.06	+5.05	+5.16	$\frac{29.6}{100}$
“ “ “ 0.2 “.	+4.90	+4.64	+4.32	+4.07	+3.84	$\frac{34.3}{100}$
“ “ “ 0.5 “.	+4.58	+3.53	+2.68	+2.04	+1.56	$\frac{52.5}{100}$

\* l = 200 mm.

Thus the changes in these three experiments were in the same sense as in the first three.

In the next set of experiments, Nos. 7, 8, and 9, the conditions were the same as in the first three, respectively. The time only was varied by extending it to 9 days.

*Experiment 7.*—At the end of the experiment the rotation of the solution was  $+4.95^\circ$  in a 200 mm. tube. The ratio of  $\frac{\text{amino N}}{\text{total N}} =$

$\frac{29}{100}$ . 10 cc. of the solution of the hydrolyzed material contained 0.0798 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{25} = \frac{-0.70^\circ \times 100}{2 \times 2.54} = -13.8^\circ.$$

*Experiment 8.*—At the end of the experiment, the rotation of the solution was  $[\alpha]_D = +3.44^\circ$  in a 200 mm. tube. The ratio of  $\frac{\text{amino N}}{\text{total N}} = \frac{35.3}{100}$ . 10 cc. of the solution of the hydrolyzed material contained 0.0763 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{25} = \frac{-0.52^\circ \times 100}{2 \times 2.43} = -10.7^\circ.$$

*Experiment 9.*—At the end of the experiment the rotation of the solution was  $[\alpha]_D = +1.26^\circ$  in a 200 mm. tube. The ratio of  $\frac{\text{amino N}}{\text{total N}} = \frac{56}{100}$ . 10 cc. of the solution of the hydrolyzed material contained 0.0784 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{25} = \frac{-0.44^\circ \times 100}{2 \times 2.47} = -8.9^\circ$$

In the next set of experiments it was aimed to ascertain more definitely the influence of temperature. In Experiments 10 and 11 the temperature was maintained at  $12.5^\circ\text{C}$ ., in Experiment 12 at  $25^\circ\text{C}$ . The duration of Experiments 10 and 11 was 12 days and in Experiment 12, 28 days. In every experiment 0.5480 gm. of the tetrapeptide was dissolved in 20 cc. of 0.5 N alkali.

*Experiment 10.*—At the end of the experiment 10 cc. of the solution of the hydrolyzed material contained 0.0983 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{25} = \frac{-0.80^\circ \times 100}{2 \times 3.13} = -12.7^\circ.$$

*Experiment 11.*—At the end of the experiment 10 cc. of the solution of the hydrolyzed material in 10 per cent hydrochloric acid contained 0.0930 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.68^\circ \times 100}{2 \times 2.96} = -11.4^\circ.$$

*Experiment 12.*—At the end of the experiment 10 cc. of the solution of the hydrolyzed material in 10 per cent hydrochloric acid contained 0.0880 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.61^\circ \times 100}{2 \times 3.16} = -9.7^\circ.$$

In the following experiment levo-alanyl-glycine anhydride was subjected to prolonged action of alkali.

*Experiment 13.*—0.400 gm. of the anhydride was dissolved in 20 cc. of 0.5 N alkali and allowed to digest at 25°C. for 15 days. At the end of the experiment 10 cc. of the hydrolyzed material in 10 per cent hydrochloric acid contained 0.0543 gm. of nitrogen. Hence the rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.43^\circ \times 100}{2 \times 1.73} = -12.4^\circ.$$

#### *Influence of Alkali on Alanine.*

Three samples of alanine each of 0.2500 gm. were dissolved, the first sample in 20 cc. of 0.1 N alkali, the second in 20 cc. of 0.2 N alkali, and the third in 20 cc. of 0.5 N alkali. The solutions provided with a little antiseptic were allowed to stand 35 days at 25°C. The respective rotations in 200 mm. tubes were +0.08°, +0.10°, and +0.16° and remained constant throughout the entire time of the experiment.



# THE IONIZATION OF PYRIMIDINES IN RELATION TO THE STRUCTURE OF PYRIMIDINE NUCLEOSIDES.

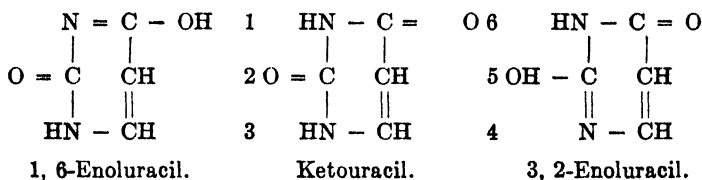
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## INTRODUCTION.

The question of the position of the sugar radical on the pyrimidine radical in uridine and cytidine has hitherto been approached by indirect methods. In previous publications from this laboratory<sup>1,2</sup> it was made clear that the choice lies only between position (3) and position (4).



Two properties of uridine argue in favor of position (3). They are first, the fact that dihydrouridine has the properties of an ordinary glucoside, and second, the fact that uridine forms a di-(phenylhydrazino) derivative.

On the other hand, the dissociation constants found in this laboratory<sup>3</sup> for uracil, uridine, and uridine phosphoric acid seemed to be in disagreement with the above view. The following (uncorrected) constants were reported.

<sup>1</sup> Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608.

<sup>2</sup> Levene, P. A., *J. Biol. Chem.*, 1925, lxiii, 653.

<sup>3</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxv, 519.

Substance.	1st $H_3PO_4$ .	2nd $H_3PO_4$ .	Aromatic hydroxyl group.	Sugar group.*
Uracil.....			9.28	(13.56†)
Uridine.....			9.17	12.52
Uridine phosphoric acid.....	1.02	5.88	9.43	(13.9)

\* It is now known that the values in the last column represent sugar groups.

† It is now known that this constant does not exist.

Thus, each of these substances was reported to have a constant above pH 12. If this value were correct for uracil, it should be concluded that this represented the ionization of the second hydroxyl group in the dienolic form of uracil. By analogy it would appear that the corresponding constants observed in uridine and in uridine phosphoric acid also represented a second hydroxyl group in the uracil radical.

These conclusions could not be correct unless the sugar linkage were in position (4), since if it were in position (3), only one hydroxyl group in the uracil radical of uridine could ionize.

However, the numerical value of the higher constant (13.56) reported for uracil was much too high to correspond to the electrostatic work<sup>4</sup> between two hydroxyl groups in the same ring. The same applies to uridine and uridine phosphoric acid.

Furthermore, the dissociation constants of glucosides<sup>5</sup> come in this pH range and suggest the possibility that in uridine and in uridine phosphoric acid, the upper constants may be due to ionization of the sugar groups.

We have therefore determined the dissociation constants of several pyrimidines (see Fig. 1) and the following conclusions were reached.

#### RESULTS AND CONCLUSIONS.

1. 1-Methyluracil and 3-methyluracil each gives *only one* constant, as is to be expected. Each has a numerical value about the same as that of phenol or succinimide, which indicates that positions (2) and (6) have almost identical intrinsic tendencies to

<sup>4</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, *xlvi*, 1251.

<sup>5</sup> Kuhn, R., and Sobotka, H., *Z. physik. Chem.*, 1924, *cix*, 65.

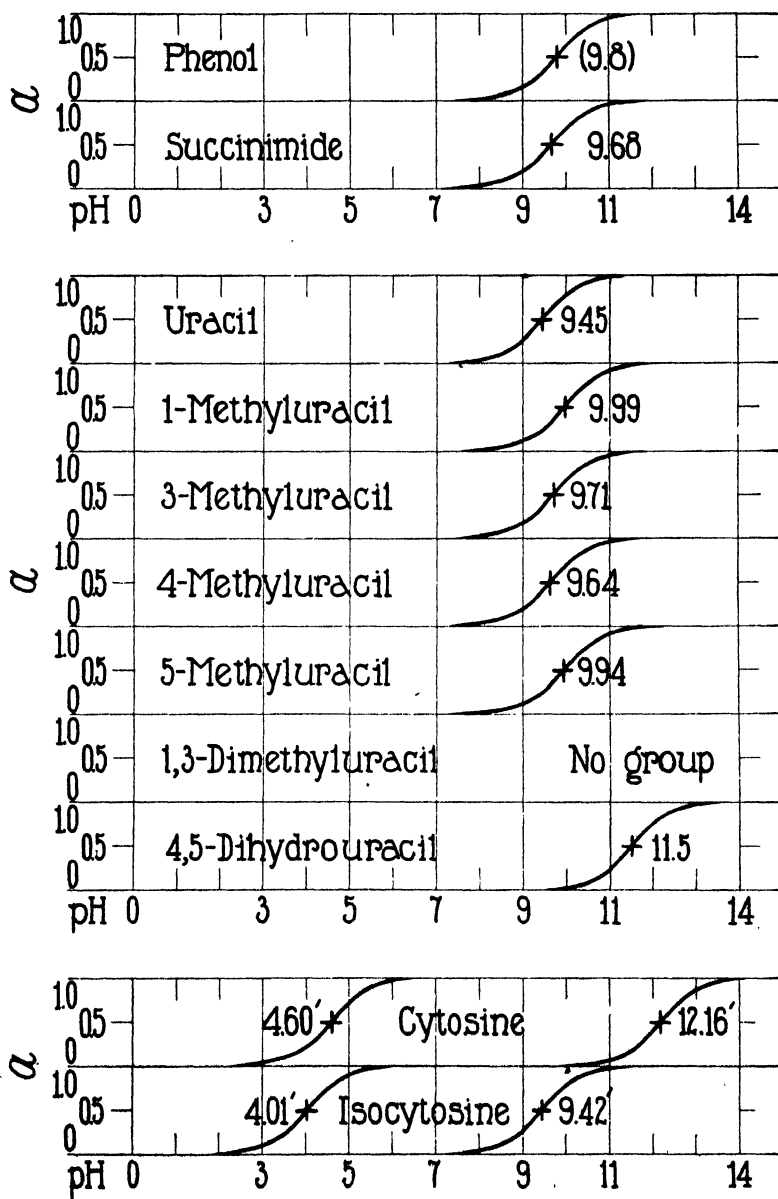


FIG. 1. Dissociation curves of pyrimidine derivatives compared with those of phenol and succinimide.



ionize, each corresponding to the typical value for an aromatic hydroxyl group. If the work of enolization were appreciable their  $pK$  values would be higher.

2. 1,3-Dimethyluracil has no ionizable group, as would be expected.

3. A redetermination of the titration data of uracil shows that it has *only one* constant (9.45 corrected). The values of  $b'$  were obtained at pH values up to 13.28 and showed that there is no upper constant. A recalculation of the previous data on uracil shows that the apparent upper constant was due to error in the activity correction (*i.e.*, only one group ionizes).

4-Methyluracil and thymine (5-methyluracil), which like uracil itself have theoretically two ionizable groups, also have *only one* constant each, the value of which corresponds with that of phenol.

This makes it evident that the enolization of one group in uracil inhibits the enolization of the other, even though each has about the same intrinsic tendency to enolize. Hence, if uracil has *only one* constant, the uracil radical in uridine should have *only one* ionizing group regardless of whether the sugar linkage is in position (3) or position (4).

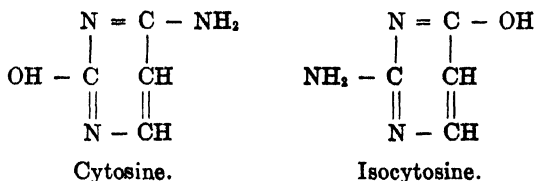
4. A recalculation of the previous data on uridine shows that it has an upper constant (as previously reported), its value being 12.54. This must be due to the ionization of a sugar group.

This is in agreement with the values 12.2, 12.33, and 12.5 for the sugar groups in inosine, inosinic acid, and adenosine, respectively, which are being reported in another article, and also with the value 12.3 previously reported for cytidine, and (13.2) for cytidine phosphoric acid previously reported as "hydroxyl" groups but which are evidently sugar groups.

Thus, the dissociation constants of uridine and its derivatives do not furnish evidence which enables one to choose between the two theories of the structure of uridine. However, these constants are important inasmuch as they do not contradict the theory of the union in position (3) of the pyrimidine.

5. Evidence for the latter theory is furnished by the dissociation constants of cytosine and cytidine.

Both cytosine and isocytosine show two constants, one for the amino group and one for the hydroxyl group (although the latter in cytosine has an abnormal value).



Since it is shown that the sugar groups in nucleosides have constants at about 12.4, and since cytosine has a constant at 12.16 corresponding to the hydroxyl in position (3), it is evident that if the sugar linkage in cytidine were in position (3) the hydroxyl group could not ionize. On the other hand, if the linkage were in position (4), we should get *two* constants in that range, the sugar constant and the hydroxyl constant.

The fact that cytidine has only *one* group ( $\text{pK}' = 12.3$ ) in that range, indicates that *the linkage is in position (3)*. Since cytidine can be converted into *uridine* the sugar linkage of the latter must be in the *same position*.

6. It is interesting to note that 4,5-dihydrouracil has an abnormally high constant at 11.5 and that the material hydrolyzes rapidly in alkaline solution. (The constant was obtained by a series of measurements extrapolated to zero time.)

#### EXPERIMENTAL.

The pH measurements were made at 25.0°C. in water-jacketed hydrogen electrode cells, using as a standard 0.1000 M HCl equals pH 1.075, and assuming constant liquid junction potential with saturated KCl.

The calculations were made as previously described<sup>6</sup> except in the alkaline range where the following procedure was adopted.

Each pH value was subtracted from<sup>7</sup>  $\text{pK}_w = 13.89$  to obtain the value of pOH. The pOH values (activity) were converted

<sup>6</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

<sup>7</sup> This value of  $\text{pK}_w = 13.89$  at 25.0°C. was obtained from the pH value (12.795) of 0.1000 M NaOH (as determined on our apparatus) and the activity value (0.81) of the hydroxyl ion given by Lewis and Randall (Lewis, G. N., and Randall, M., *Thermodynamics*, New York and London, 1923, 382). It agrees well with the value of Michaelis (13.895) for 25°C.

TABLE I.

Values of  $\log \tau_H = \log \frac{h}{H}$  given below were calculated from the activity values of H ion given by Lewis and Randall and by Scatchard\* with a saturated KCl junction which agree closely with (unpublished) values obtained in this laboratory.

Also values of  $\log \tau_{OH} = \log \frac{oh}{OH}$  were calculated from the activity values of OH ion given by Lewis and Randall and (at the higher concentrations) from data obtained in this laboratory on the pH values of NaOH solutions (taking the value of Lewis and Randall for ionic strength of 0.100 as being correct).

$H$  and  $OH$  refer to activities,  $h$  and  $oh$  refer to concentrations,  $\mu$  = ionic strength.

$\log \tau_H$	$\mu$	$\log \tau_{OH}$	$\mu$	$\log \tau_{OH}$	$\mu$
0.01	0.001	0.01	0.001	0.11	0.17
0.02	0.004	0.02	0.004	0.12	0.21
0.03	0.007	0.03	0.007	0.13	0.27
0.04	0.014	0.04	0.013	0.14	0.34
0.05	0.025	0.05	0.019	0.15	0.42
0.06	0.041	0.06	0.030	0.16	0.50
0.07	0.070	0.07	0.048	0.17	0.60
0.08	0.22	0.08	0.070	0.18	0.70
		0.09	0.097	0.19	0.83
0.07	0.80	0.10	0.130	0.20	1.00
0.06	1.00				

into  $p_{oh}$  values (concentration) by use of Table I in which values of  $\log \tau_{OH} = p_{OH} - p_{oh} = \log \frac{oh}{OH}$  may be obtained.

From these values of  $p_{oh}$  we may calculate

$$\frac{b-a}{c} + \frac{h-oh}{c}$$

which becomes in alkaline solution:

$$\therefore \quad b-a \quad \frac{oh}{c}$$

The results are given in Tables II to XI. As stated above, dihydrouracil hydrolyzed rapidly and its  $pG'$  value was obtained by

\* Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 641.

extrapolation to zero time. 5-Nitouracil was found to poison the electrodes and could not be titrated.

The values marked "Deviation" in the last columns of Tables II to VII represent the error in the pH value which would give the experimental values of  $b'$ , assuming that the correct value of  $b'$  in this range is 1.000. In general the deviations of  $b'$  from unity in this pH range are more likely to be due to slight impurity than to error in pH. It is evident that none of the substances in Tables II to VII has measurable constants above pH 10.

The titration constants ( $pG'$ ) in these substances are all equal to the dissociation constants ( $pK'$ ) and the corrected constants ( $pG$  or  $pK$ ) are corrected for activity as previously described.<sup>6</sup>

TABLE II.  
*Succinimide. (0.0200 Molar.)*

pH	$\frac{b-a}{c}$	$b' = \alpha$	$pG'$	$pG$	Deviation.  <i>pH units</i>
5.60	0*	0			
8.72	0.100	0.100	9.67	9.69	
9.02	0.200	0.200	9.62	9.65	
9.47	0.400	0.398	9.65	9.69	
9.82	0.600	0.595	9.65	9.70	
10.20	0.800	0.789	9.63	9.68	
11.47	1.200	0.982	(9.72)		0.04
11.89	1.500	0.925			0.06
12.17	2.000	0.880			0.05
Average.....			9.64	9.68	

\* The mother solution was made up from pure substance.

TABLE III.

*Uracil.*

pH	$\frac{b-a}{c}$	$c$	$b' = \alpha$	pG'	pG	Deviation.
						<i>pH units</i>
3.68	0	0.0800	0			
9.12	0.375	0.0800	0.375	9.34	9.46	
9.32	0.500	0.0800	0.500	9.32	9.44	
9.53	0.625	0.0800	0.625	9.31	9.43	
9.83	0.750	0.0800	0.750	9.35	9.47	
11.11	1.000*	0.0800	0.975	(9.51)		
12.43	1.500	0.0800	0.934			0.04
12.71	2.000	0.0800	0.936			0.02
11.19	1.000*	0.250	0.990			
12.45	1.200	0.250	1.004			
12.75	1.400	0.250	1.000			
12.91	1.600	0.250	1.008			
13.02	1.800	0.250	1.036			0.02
13.11	2.000	0.250	1.040			0.02
13.28	2.600	0.250	1.112			0.03
Average.....				9.33	9.45	

\* The mother solutions were made up with 1.000 equivalent of NaOH.

TABLE IV.

*1-Methyluracil. (0.0400 Molar.)*

pH	$\frac{b-a}{c}$	$b' = \alpha$	pG'	pG	Deviation.
					<i>pH units</i>
6.17	0				
9.42	0.250	0.250	9.90	9.99	
9.89	0.500	0.497	9.89	9.98	
10.38	0.750	0.741	9.92	10.01	
11.19	1.000*	0.942	(9.97)		
12.43	2.000	0.958			0.02
Average.....			9.90	9.99	

\* The mother solution was made up with 1.000 equivalent of NaOH.

TABLE V.  
*3-Methyluracil. (0.0380 Molar.)*

pH	$\frac{b-a}{c}$	$b' = \alpha$	pG'	pG	Deviation.
					<i>pH units</i>
7.43	0	0			
9.19	0.263	0.263	9.63	9.71	
9.67	0.523	0.522	9.63	9.71	
10.19	0.790	0.784	9.64	9.72	
11.25	1.053*	0.982	(9.50)		
11.94	1.316	0.969			0.02
12.45	2.105	0.949			0.01
12.77	3.158	0.771			0.04
12.89	4.211	0.986			0.00
Average.....			9.63	9.71	

\* The mother solution was made up with 1.000 equivalent of NaOH.

TABLE VI.  
*4-Methyluracil.*

pH	$\frac{b-a}{c}$	$c$	$b' = \alpha$	pG'	pG	Deviation.
						<i>pH units</i>
7.29	0	0.0400				
9.09	0.250	0.0400	0.250	9.57	9.66	
9.55	0.500	0.0400	0.499	9.55	9.64	
9.99	0.750	0.0400	0.746	9.53	9.62	
10.68	1.000*	0.0400	0.982			
11.73	1.250	0.0400	1.047			0.09
12.40	2.000	0.0400	1.030			0.01
12.72	3.000	0.0400	0.870			0.03
11.23	1.000*	0.0800	0.967			
12.16	1.250	0.0800	0.964			
12.43	1.500	0.0800	0.957			
12.82	2.250	0.0800	0.875			0.04
13.09	3.500	0.0800	0.824			0.03
13.22	4.250	0.0800	0.562			0.05
Average.....				9.55	9.64	

\* The mother solutions were made up with 1.000 equivalent of NaOH.

TABLE VII.  
*Thymine (5-Methyluracil). (0.0800 Molar.)*

pH	$\frac{b-a}{c}$	$b' = \alpha$	pG'	pG	Deviation.
					<i>pH units</i>
9.85	0.500	0.499	9.85	9.97	
9.97	0.600	0.598	9.80	9.92	
10.17	0.700	0.697	9.81	9.93	
10.28	0.750	0.746	9.81	9.93	
11.25	1.000*	0.966			
12.16	1.250	0.964			0.06
12.44	1.500	0.946			0.05
12.76	2.000	0.806			0.08
13.02	3.000	0.778			0.04
Average .....			9.82	9.94	

\* The mother solution was made up with 1.000 equivalent of NaOH.

TABLE VIII.  
*1,3-Dimethyluracil. (0.0400 Molar.)*

pH	$\frac{b-a}{c}$	$b' = \alpha$
4.84	0*	
9.96	0.100	0.097†
10.81	0.200	0.178†
11.80	0.400	0.177†
12.09	0.600	0.145†
12.25	0.800	0.128†
12.36	1.000	0.133†
12.61	1.600	0.023†

\* The mother solution was made up from pure substance.

† The deviations of  $b'$  from zero do not correspond to the presence of an ionizable group, but probably result from impurity.

TABLE IX.  
4,5-Dihydrouracil. (0.0400 Molar.)

A. Values Obtained about 15 Minutes after Making up Solutions.

pH	$\frac{b-a}{c}$	$b' = \alpha^*$	pG'
1.97	-0.250		
7.30	0*	0	
10.82	0.250	0.227	11.35
11.25	0.500	0.436	11.36
11.63	0.750	0.592	(11.47)
11.83	1.000	0.745	11.37

B. Values Obtained at Varying Intervals after Making up Solutions.

$\frac{b-a}{c}$	Time,	pH	$b'$	pG'	pG
	min.				
0.500	31	11.29			
0.500	24	11.32			
0.500	15	11.35			
0.500	0†	11.38	0.429	11.50	11.56
0.750	35	11.54			
0.750	26	11.60			
0.750	17	11.63			
0.750	0†	11.65	0.616	11.45	11.52
Average.....					11.5

\* The mother solution was made up from pure substance.

† Zero values obtained by extrapolation.



TABLE X.  
*Cytosine.*

pH	$\frac{b-a}{c}$	$c$	$b'$	$\alpha_1$	$\alpha_2$	pG <sub>1</sub> '	pG <sub>2</sub> '
4.02	-0.800	0.0200	-0.800	0.200		4.62	
4.41	-0.600	0.0200	-0.600	0.400		4.59	
4.80	-0.400	0.0200	-0.400	0.600		4.62	
5.19	-0.200	0.0200	-0.200	0.800		4.59	
6.91	0*	0.0200	0				
11.11	0.200	0.0200	0.113		0.113		(12.00)
11.48	0.400	0.0200	0.187		0.187		(12.11)
11.65	0.600	0.0200	0.277		0.277		(12.06)
11.83	0.800	0.0200	0.312		0.312		(12.17)
11.59	0.300	0.0800	0.230		0.230		12.11
11.76	0.400	0.0800	0.294		0.294		12.14
11.89	0.500	0.0800	0.354		0.354		12.18
11.99	0.600	0.0800	0.415		0.415		12.14
12.10	0.700†	0.0800	0.461		0.461		12.17
12.19	0.800	0.0800	0.500		0.500		12.19
12.33	1.000	0.0800	0.586		0.586		12.18
12.49	1.300	0.0800	0.688		0.688		12.15
12.78	2.000	0.0800	0.750		0.750		(12.30)
Average.....						4.60	12.16

\* The first mother solution was made up from pure substance.

† The second mother solution was made up with 0.700 equivalent of NaOH.

TABLE XI.  
*Isocytosine. (0.0800 Molar.)*

pH	$\frac{b-a}{c}$	$b'$	$\alpha_1$	$\alpha_2$	pG <sub>1</sub> '	pG <sub>2</sub> '
2.35	-1.000*	-0.940	0.060			
3.49	-0.750	-0.746	0.254		3.96	
4.03	-0.500	-0.499	0.501		4.03	
4.52	-0.250	-0.250	0.750		4.04	
7.34	0					
8.99	0.250	0.250		0.250		9.47
9.37	0.500	0.500		0.500		9.37
9.89	0.750	0.750		0.750		9.41
10.80	1.000	0.999		0.999		
Average.....					4.01	9.42

\* The mother solution was made up with 1.000 equivalent of HCl.

## SUMMARY.

The dissociation constants of a number of pyrimidine derivatives have been obtained and lead to the following conclusions.

Positions (2) and (6) in uracil have approximately equal tendencies to enolize and ionize, and the work of enolization is small. However, the enolization of one position inhibits the enolization of the other position and uracil has only one dissociation constant. Hence, the data on uracil and uridine fail to indicate the position of the sugar linkage in uridine. There is also only one dissociation constant in 4-methyl- and in 5-methyluracil.

Both cytosine and isocytosine show two constants (only one enolization being required) although the hydroxyl constant of the former has an abnormal value. If the sugar linkage in cytidine were in position (4), the sugar group and also the hydroxyl in position (3) would ionize in the alkaline range. However, since there is only one constant for cytidine in the alkaline range, the linkage must be in position (3).

Uridine must also have its linkage in position (3) since it may be formed from cytidine.



# THE EFFECT OF IONIZATION UPON OPTICAL ROTATION OF NUCLEIC ACID DERIVATIVES.

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## *Theory.*

It is well known that optically active free acids have optical rotations different from those of their salts.

1. It is to be expected that each molecular or ionic species should have its individual optical rotation and that the observed value in a given solution should be the sum of the effects of the various constituents:

$$[M] = [M_A] A + [M_B] B + [M_C] C + \text{etc.} \quad (1)$$

where  $A$ ,  $B$ , and  $C$  are the fractions in various molecular or ionic forms and  $[M_A]$ ,  $[M_B]$ , and  $[M_C]$  are the respective molecular rotations.

In an unbuffered pH range there is only one molecular or ionic form in appreciable quantity. In a buffered pH range, where the titration constants are at least 3 pH units apart, there are two forms in appreciable quantities. In the case of a monovalent acid, we may write<sup>1</sup>

$$G' = H \frac{\alpha}{1 - \alpha} = H \frac{m}{u} \quad (2)$$

where

$$m = \alpha = 1 - u \quad (3)$$

---

<sup>1</sup>For notation and significance of titration constant ( $G'$ ) see Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

From equations (1) and (3) we may write

$$[M] = [M_u] u + [M_m] m = [M_u] (1 - m) + [M_m] m \quad (4)$$

hence

$$\alpha = \frac{[M] - [M_u]}{[M_m] - [M_u]} \quad (5)$$

and from equation (2) we get:

$$G' = H \frac{\alpha}{1 - \alpha} = H \frac{[M] - [M_u]}{[M_m] - [M]} \quad (6)$$

from which it follows that if we plot  $\alpha$  against  $[M]$  we should get a straight line curve.

Similarly for polyvalent substances having titration constants at least 3 pH units apart, if we plot molecular rotation  $[M]$  against corrected equivalents of base:

$$b' = \frac{b - a + h - oh}{n} = \alpha_1 + \alpha_2 + \alpha_3 + \text{etc.} - y \quad (7)$$

we should get a curve which is a straight line from  $b' = 0$  to  $b' = 1$  and which is straight from  $b' = 1$  to  $b' = 2$ , etc. There should be a sharp break at each integral value of  $b'$  (see Fig. 1). The intercept at  $b' = 0$  is the molecular rotation of the undissociated form. The intercept at  $b' = +1$  is the rotation of the monoanion, etc.

In the case of substances with overlapping buffer ranges (*i.e.*, titration constants less than 3 pH units apart) the curves should not have sharp breaks at the integral values of  $b'$  but should be rounded off. (We have obtained no data on this type of substance.)

2. In dealing with substances such as purines or pyrimidines which occur in tautomeric forms but which ionize in only one of these forms, the question arises as to whether the work of tautomerization (or else whether the equilibrium in such a change) can influence the relation found above.

We are particularly concerned with the ionization of the hydroxyl resulting from enolization. If  $u_e$  and  $u_k$  are the fractions

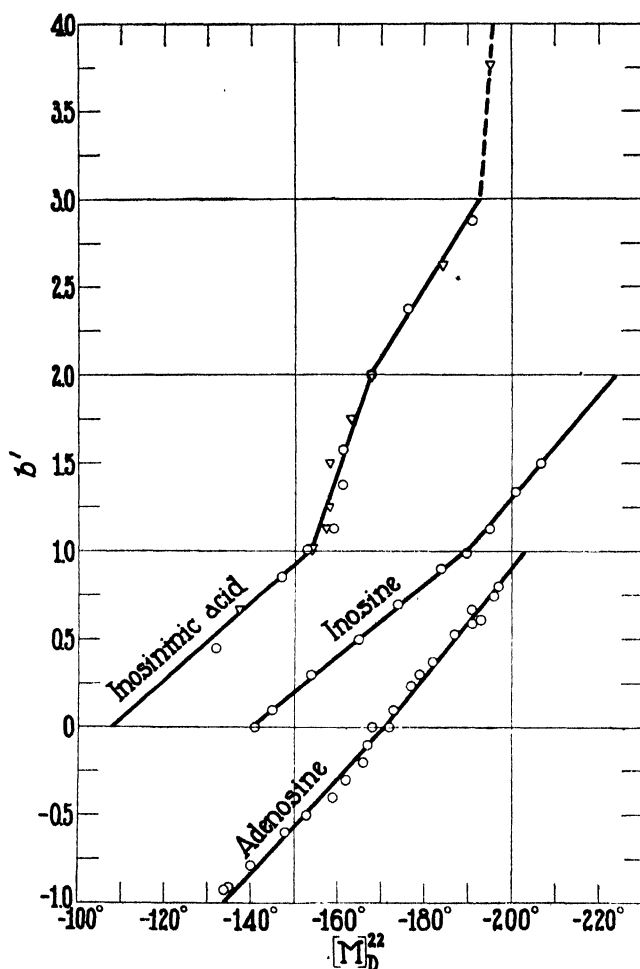


FIG. 1. Molecular rotations plotted against degree of dissociation.

in the undissociated enol form and the (undissociated) keto form, respectively, we may write for the true titration constant:

$$G' = H \frac{m}{u_e} = H \frac{m}{1 - m - u_k} \quad (8)$$

where

$$m + u_e + u_k = m + (1 + R)u_e = 1 \quad (9)$$

and

$$R = \frac{u_k}{u_e},$$

and for the observed titration constant:

$$G' \text{ obs.} = H \frac{m}{u_e + u_k} = \frac{G'}{1 + R} \quad (10)$$

Hence, if  $G' \text{ obs.}$  is found to be constant the ratio  $R$  must also be constant and equal to

$$R = \frac{u_k}{u_e} = \frac{G'}{G' \text{ obs.}} - 1 = \frac{1 - m}{u_e} - 1 \quad (11)$$

As in equation (4) we may write:

$$\begin{aligned} [M] &= [M_m] m + [M_e] u_e + [M_k] u_k \\ &= [M_m] m + ([M_e] + [M_k] R) u_e \\ &= [M_m] m + Q (1 - m) \end{aligned} \quad (12)$$

where

$$Q = \frac{[M_e] + [M_k] R}{1 + R} = \frac{[M_e] u_e + [M_k] u_k}{u_e + u_k} \quad (13)$$

$$m = \frac{[M] - Q}{[M_m] - Q} \quad (14)$$

From this it is obvious that this data will also give a straight line curve when we plot  $[M]$  against  $b'$ . The intercepts at  $b' = 0$  and  $b' = 1$  will be  $Q$  and  $[M_m]$  respectively.

$$G' \text{ obs.} = H \frac{m}{1 - m} = H \frac{[M] - Q}{[M_m] - [M]} \quad (15)$$

### Results.

We have studied three compounds, inosine, inosinic acid, and adenosine. These have two, three, and two ionizing groups respectively, and the titration constants are at least 3 pH units apart in each substance.

Solutions were made up containing various quantities of strong acid or alkali. The optical rotations were observed and the pH values were obtained. From the pH values the values of  $b'$  were calculated (equation (7)) and the molecular rotations  $[M]$  were plotted against  $b'$  in Fig. 1.

It will be observed that the curves are approximately straight lines between the integral values of  $b'$  and that there are sharp breaks in the curves at each integral value of  $b'$  (as necessitated by the fact that the  $pG'$  values are at least 3 pH units apart in each substance).

TABLE I.

## A.

Titration constants (equal in each of these cases to the dissociation constants) not corrected for activity.

Substance.	Primary phosphoric group.	Aromatic amino group.	Secondary phosphoric group.	Aromatic hydroxyl group.	Sugar group.
Inosinic acid.....	1.54		6.04	8.88	(12.2)
Inosine.....				8.75*	12.33
Adenosine.....		(3.3)*			12.5

\* Previously obtained values were 8.71 for the hydroxyl in *inosine* and 3.44 for the amino group in *adenosine*.

## B.

Molecular rotations of the various molecular and ionic species as given by the intercepts of the curves with the integral values of  $b'$ .

Substance.	$b' = -1$	$b' = 0$	$b' = 1$	$b' = 2$	$b' = 3$	$b' = 4$
Inosinic acid.....		-108°	-154°	-167.5°	-193°	(-196°)
Inosine.....		-140°	-190°	-224°		
Adenosine.....	-134°	-171°	-203°			

The molecular rotations of the various molecular or ionic species are obtained from the intercepts with the integral values of  $b'$  (extrapolating when necessary). The results are given in Table I.

The rotations of the second group in inosinic acid were not as satisfactory as the others due to turbidity of the solution.

Adenosine was found to hydrolyze in strong acid or alkali,



particularly the latter, on slight warming. Five experiments were run. We give only the data from the last, in which considerable care was taken to avoid hydrolysis.

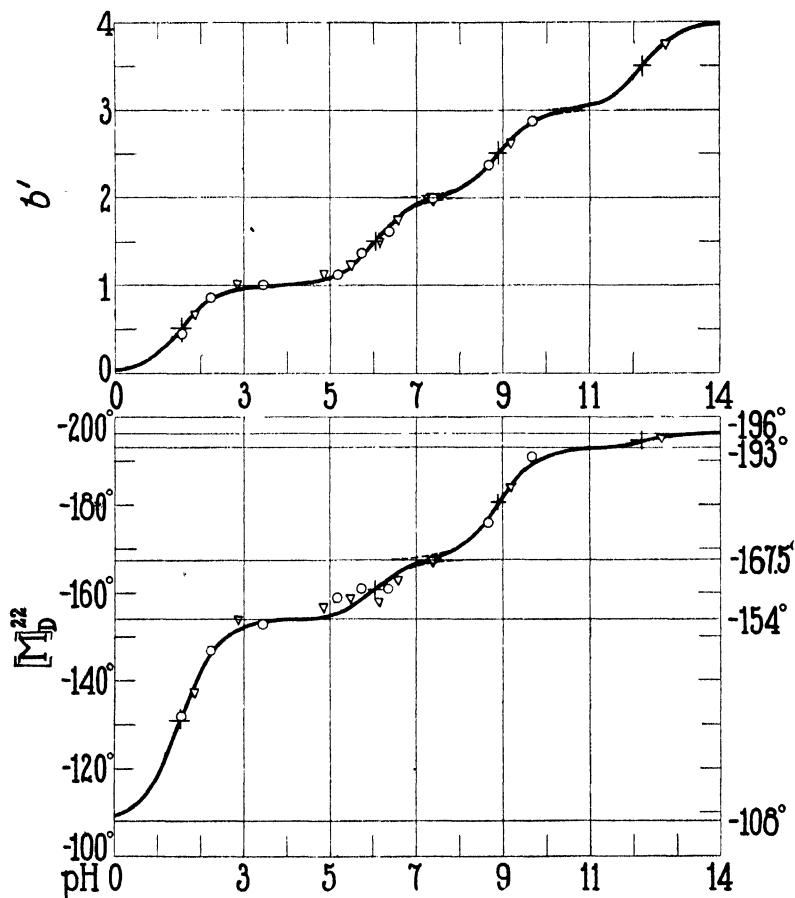


FIG. 2. Comparison of the rotation-pH curve (lower curve) and the titration curve (upper curve) of inosinic acid.

We present Fig. 2 to illustrate the relation between rotation and pH, compared with the titration curve ( $b'$  and pH) in inosinic acid.

## EXPERIMENTAL.

Mother solutions of the substances were made up with the indicated amount of acid or alkali to twice the indicated strength, and 5 cc. samples were diluted to 10 cc. with the requisite quantities of acid or alkali. The inosinic acid was weighed out as the barium salt ( $7.5 \text{ H}_2\text{O}$ ) and treated with an exact equivalent of  $\text{Na}_2\text{SO}_4$  to precipitate the barium.

The rotations were observed in 2 dm. open tubes (fitted with ground glass covers) holding 4 cc. and the pH measurements were made at  $25^\circ\text{C}$ . in water-jacketed hydrogen electrode cells. As a standard, the pH of 0.1000 M HCl was taken as 1.075, instead of 1.090, the value previously used in this laboratory. The saturated KCl liquid junction was assumed constant. The results are given in Tables II to IV.

TABLE II.  
*Inosinic Acid (Concentration 0.0800 Molar).*  
*Experiments A and B.*

Experiment	$[\text{M}]_D^{25}$	pH	$\frac{b-a}{c}$	$b'$	$\text{pG}_1'$	$\text{pG}_2'$	$\text{pG}_3'$	$\text{pG}_4'$
A	$-132^\circ$	1.57	0.125	0.450	(1.65)			
B	$-137.5^\circ$	1.87	0.500	0.665	1.52			
A	$-147^\circ$	2.23	0.750	0.852	1.56			
A	$-153^\circ$	3.45	1.000	1.004				
B	$-154^\circ$	(2.86)	1.000	1.017				
B	$-157^\circ$	4.86	1.125	1.125				
A	$-159^\circ$	5.18	1.125	1.125		6.03		
B	$-153^\circ$	5.47	1.250	1.250		5.95		
A	$-161^\circ$	5.71	1.375	1.375		5.93		
B	$-158^\circ$	6.11	1.500	1.500		6.11		
A	$-161^\circ$	6.36	1.625	1.625		6.14		
B	$-163^\circ$	6.57	1.750	1.750		6.09		
A	$-167.5^\circ$	7.38	2.000	2.000				
B	$-167.5^\circ$	7.38	2.000	2.000				
A	$-176^\circ$	8.66	2.375	2.375			8.88	
B	$-184^\circ$	9.18	2.625	2.625			8.96	
A	$-191^\circ$	9.66	2.875	2.875			8.81	
B	$-195^\circ$	12.67	4.500	3.76				12.2
Average values.....					1.54	6.04	8.88	12.2

TABLE III.  
*Inosine (Concentration 0.0930 Molar).*

$[M]_D^{22}$	pH	$\frac{b-a}{c}$	$b'$	$pG_1'$	$pG_2'$	$pG_3$
-141°	4.64	0	0			
-145°	7.81	0.100	0.107	8.73		
-154°	8.42	0.300	0.323	8.74		
-165°	8.43	0.500	0.538	8.76		
-174°	9.24	0.700	0.753	8.76		
-184°	9.87	0.900	0.967	(8.62)		
-190°	10.67	1.000	1.068			
-195°	11.71	1.200	1.208		12.29	12.44
-201°	12.25	1.600	1.431		12.37	12.54
-207°	12.52	2.000	1.613		12.32	12.52
Average $pG'$ values.....				8.75	12.3	
(Corrected) $pG$ values.....				8.85		12.5

TABLE IV.  
*Adenosine (Concentrations Given in Second Column).*

$[M]_D^{22}$	$c$	$\frac{b-a}{c}$	pH	$b'$	$pG_1'$	$pG_2'$
-134°	0.100	-1.2	1.64	-0.924	3.25	
-135°	0.100	-1.0	2.10	-0.904	3.11	
-140°	0.100	-0.8	2.76	-0.779	3.31	
-148°	0.100	-0.6	3.15	-0.599	3.29	
-153°	0.100	-0.5		-0.500		
-159°	0.100	-0.4	3.56	-0.400	3.39	
-162°	0.0500	-0.3		-0.300		
-166°	0.0500	-0.2	4.09	-0.200	3.49	
-167°	0.0500	-0.1		-0.100		
-168	0.0500	0		0		
-172°	0.0500	0	10.43	0		
-173°	0.0500	0.1	11.70	(0.100)		
-177°	0.0500	0.5	11.91	0.236		12.42
-179°	0.0500	0.7	12.09	0.300		12.46
-182°	0.0500	0.9	12.21	0.374		12.43
-187°	0.100	1.1	12.55	0.524		12.51
-191°	0.100	1.5	12.75	0.588		12.59
-193°	0.100	1.7	12.82	0.603		12.63
-191°	0.100	1.9	12.87	0.670		12.56
-196°	0.100	2.5	13.04	(0.740)		(12.79)
-197°	0.100	3.5	13.20	(0.80)		(12.87)
Average.....					(3.3)	12.5

The previously reported value of  $pG_1'$  was 3.45 and was more accurate than the value (3.3) given here.

## SUMMARY.

1. It is to be expected that if each molecular or ionic species of a substance has its individual molecular rotation, then a plot of *molecular rotation* [M] against *corrected equivalents of base*,  $b'$ , should give straight line curves between the integral values of  $b'$ .

2. If the titration constants are more than 3 pH units apart there should be a sharp break at each integral value of  $b'$  and these points indicate the molecular rotations of the various ionic species. If the constants are less than 3 pH units apart, the corners should be rounded off.

3. Three substances, inosine, inosinic acid, and adenosine were studied. They gave *straight line curves with sharp breaks* at the integral values of  $b'$  (as would be expected from their titration constants).

4. Inosine and inosinic acid contain aromatic hydroxyl groups which can ionize only in the enolic form. These groups gave straight line curves.

5. All three substances have ionizable sugar groups with titration constants above pH 12.



# THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES.

## III. ENZYME HYDROLYSIS OF DIPEPTIDES AND TRIPEPTIDES.

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### INTRODUCTION.

In two previous papers we have reported on the hydrolysis by acid<sup>1</sup> and by enzyme<sup>2</sup> (erepsin) of the following dipeptides: glycyl-glycine (GG), sarcosyl-glycine (SG), glycyl-sarcosine (GS), sarcosyl-sarcosine (SS).

It was found that each peptide simultaneously hydrolyzed and formed anhydride at rates dependent upon the values of  $\left(\frac{K_a}{K_b}\right)^2$  where  $K_a$  and  $K_b$  are the acid and basic dissociation<sup>3</sup> constants of the groups involved in the respective linkages (*i.e.*, the strength of the peptide bond is determined by amino acid constants, while

<sup>1</sup> Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, 1924, lxi, 445.

<sup>2</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1924-25, lxii, 711.

<sup>3</sup> In the above mentioned papers the term "dissociation" was used synonymously with "ionization" of both acid and basic groups, and we wrote  $(K_a K_b)$  to represent the product of the corresponding constants.

We have since that time reversed our notation with regard to basic (amino) groups and define "dissociation" as the "effect of ionization of the acid groups and hydrolysis of the salts of basic (amino) groups" (see Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239, 1251). By this definition  $\left(\frac{K_a}{K_b}\right)$  is proportional to the product of the ionization constants, and represents the ratio of the acid and basic dissociation constants according to the new definition. This change in definition was necessary in order to deal with titration data of complex substances. We regret the confusion which it may produce.

the strength of the anhydride bond is determined by the peptide constants).

The data agreed with the conclusions that erepsin attacks the neutral molecule of peptide, and that erepsin has a dissociation constant at about  $pK' = 7$ , being active on the alkaline side.

Other dipeptides and some tripeptides have since been prepared. We desired to determine the following facts.

1. Whether these peptides agree in general with the laws which applied to the dipeptides studied before.

2. As a result of a study of the nature of dissociation constants<sup>4</sup> it seems probable that the stability of each peptide or anhydride bond should depend not upon the *dissociation* constants (nor the ionization constants) but rather upon the *intrinsic* constants of the groups involved. These should be proportional to the ionization constants in comparing the compounds studied before, but would not be proportional when we compare tripeptides with dipeptides.

3. If it is the neutral molecule which is acted upon, then asparagyl-glycine should not be hydrolyzed at all, since it is negatively charged in the range of action of erepsin.

#### OBSERVATIONS AND DISCUSSION.

1. The following peptides were hydrolyzed with erepsin at 32°C.: glycyl-glycine (GG), alanyl-glycine (AG), glycine-alanine (GA), alanyl-alanine (AA), methylalanyl-glycine (<sup>o</sup>AG), methylalanyl-alanine (<sup>o</sup>AA), sarcosyl-alanine (SA), and asparagyl-glycine (ApG), also glycyl-amide (GAm).

The results may best be observed by inspection of Fig. 1.

From the dissociation constants of alanine and of alanyl-alanine<sup>5</sup> (AA) we calculate for the hydrolysis of AA, that  $a' =$

<sup>4</sup> *Dissociation* constants represent the *steps* in dissociation according to the classical conception (providing we reverse our definition for the basic groups). *Intrinsic* constants, on the other hand, represent the tendencies of the individual chemical *groups* to dissociate and are independent of the electrostatic work between the groups. Dissociation constants involve not only the intrinsic constants but also this electrostatic work.

<sup>5</sup> For AA,  $pK_1' = 3.17$  and  $pK_2' = 8.42$  (standard, 0.100 M HCl = pH 1.075).

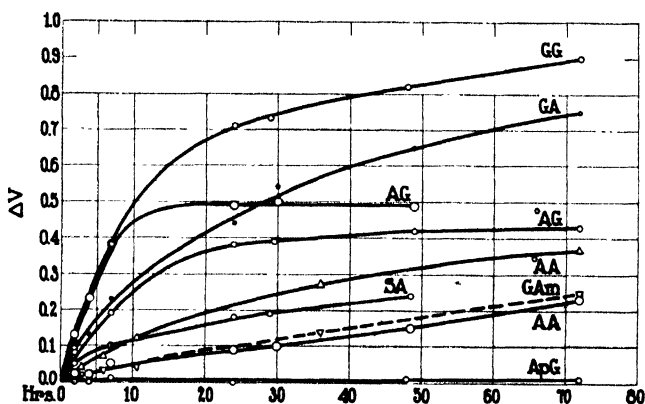


FIG. 1. Hydrolysis curves of dipeptides with erepsin.

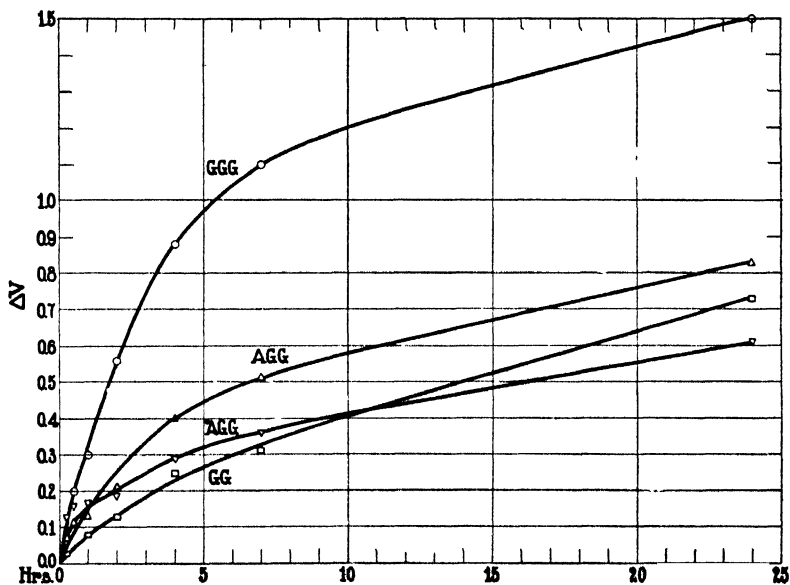


FIG. 2. Hydrolysis curves of tripeptides with erepsin.

0.33 and  $k = 0.85$  that of GG. From the experimental data we find that  $a' = 0.33$  and  $k = 0.4$  that of GG (uncorrected for steric hindrance) or  $k = 0.8$  (with a correction factor of 2).

The constants of AG and GA were not determined. It is obvious that they should fall between GG and AA which agrees



with the hydrolysis experiments. °AG has a curve slightly below AG as would be expected, and SA is about the same as AA, as we would also expect.

Therefore, these compounds agree with the laws which applied to the peptides previously studied. The behavior of asparagylglycine (ApG) will be discussed below.

2. The curves of the following tripeptides, glycyl-glycyl-glycine (GGG), alanyl-glycyl-glycine (AGG), and methylalanyl-glycyl-glycine (°AGG), are plotted in Fig. 2, while sarcosyl-glycyl-glycine (SGG) was too insoluble to be studied.

If we calculate the strength of the bonds of GGG from the dissociation constants we find, on the basis of  $\left(\frac{K_a}{K_b}\right)$  that it should split 43 times as fast as GG (one bond 37 times as fast and the other 6 times). On the basis of  $\left(\frac{K_a}{K_b}\right)^2$  we find that GGG should split 1500 times as fast as GG. The observed rate of hydrolysis is 9 times as fast as GG. (This value would be a little higher if corrected for the effect of pH upon the enzyme activity.)

We are led to conclude that the stability of each peptide bond is a function of the *intrinsic* constants of the groups involved in the linkage. This seems more logical since the *intrinsic* constants represent the affinity of the groups for electrons while the *dissociation* constants involve, in addition, electrostatic work between groups as a result of ionization. We cannot determine the *intrinsic* constants but we predict that  $pK_o^A$  (for the acid groups) in GG is slightly higher than in glycine while  $pK_o^B$  (for the basic groups) is slightly lower in GG than in glycine. If we could determine these constants, we would probably find that the stability of each bond is proportional to  $\left(\frac{K_o^A}{K_o^B}\right)$ .

This is of significance in the hydrolysis of proteins. A chain of similar amino acids should have equal probability of hydrolysis (with acid) at all peptide bonds (except the two end ones which should be more stable). It should, therefore, be impossible to systematically degrade protein by partial hydrolysis. A chain of dissimilar amino acids would, however, favor hydrolysis more at some points than at others.

It will also be observed that the ratios of the rates of hydrolysis

of AGG to AG and of  $^{\circ}$ AGG to  $^{\circ}$ AG are roughly the same as GGG to GG.

It is to be expected that the higher simple<sup>6</sup> peptides should have about the same tendencies to hydrolyze as the tripeptides since their intrinsic constants should be practically identical. The *actual* rates would depend upon the pH of the solutions. Higher peptides have lower  $pK_2'$  values, hence their solutions would be less favorable to the action of erepsin.

3. The assumption that the neutral molecule of peptide is attacked by erepsin agrees with the experiments of Dernby and our earlier observations. If this is correct, there should be no hydrolysis of asparagyl-glycine (ApG) since this substance is negatively charged throughout the whole range of action of erepsin.<sup>7</sup>

Our experiments showed that this is true. A solution of asparagyl-glycine with erepsin showed a constant titration value over a period of 5 days. That this was not due to simultaneous splitting and anhydride formation was shown by the fact that the final solution showed no change in titration value after standing 24 hours with an equivalent of NaOH. If anhydride had been present it would have hydrolyzed.

This therefore supports the view that erepsin cannot act upon peptide bonds in the proximity of negatively charged (anion) groups. In order to determine whether erepsin can act upon cations we prepared some glycyl-amide. This was found to hydrolyze slowly with erepsin (while a blank without enzyme showed no change).

<sup>6</sup> By "simple" peptides we mean those composed only of divalent amino acids and which, therefore, have only two ionizable groups: an amino group on one end of the chain and a carboxyl group on the other.

<sup>7</sup> If, as appears to be the case, erepsin is an acid which has a  $pK$  at about 7, and is active only in the ionized state (above pH 7) and is active upon amide linkings of neutral or positively charged molecules (as our experiments show) and not upon negatively charged molecules, it can never attack ApG since this substance has a single negative charge above pH 3.53 and two negative charges above 9.07.

This agrees with the observation in another laboratory that glycyl aspartic acid (GAp) does not hydrolyze with erepsin. GAp should have nearly the same  $pG$  values as ApG.

The apparent value of erepsin of  $pK = 7$  is interesting since the only ordinary acid group which ionizes at this point is a secondary phosphoric group. This would suggest that erepsin is a monophosphoric ester.

It has already been shown that erepsin acts upon the neutral molecule of the simple (divalent) dipeptides. We have now shown that it cannot act upon a negatively charged peptide but will hydrolyze a positively charged amide. It would appear that erepsin is itself negatively charged (above pH 7) and is repelled by negative groups but may attack peptide bonds in neutral or positively charged molecules.

#### EXPERIMENTAL.

The erepsin solution<sup>8</sup> was prepared as described in the second paper. The method of hydrolysis was the same as used before except that 2 cc. of enzyme solution were used (instead of 5 cc.) and the temperature was 31°C. (instead of 40°C.) for the dipeptides, except <sup>o</sup>AA and GAM, while the tripeptides were hydrolyzed at 25°C.

In each experiment GG was also hydrolyzed in order to permit comparison between the data of experiments performed under differing conditions. The results are given in Tables I to III.

#### *Preparation of Materials.*

*Preparation of Methylalanyl-Glycine.*—5 gm. of bromopropionyl-glycine were allowed to stand 2 days at room temperature with 15 cc. of 31 per cent aqueous methylamine and then concentrated under diminished pressure until crystallization began. About 50 cc. of absolute alcohol were added, and the syrup heated on the water bath until crystallization was complete. The yield was 3 gm. The material was purified by dissolving it in about 5 cc. of hot water and adding an equal volume of hot absolute alcohol. A crystalline precipitate was immediately formed. A second crop was obtained by adding more absolute alcohol to the filtrate. It melted at 237°C. with decomposition. For analysis it was dried at 100°C. over sulfuric acid under diminished pressure.

0.1000 gm. substance required (Kjeldahl) 12.50 cc. 0.1 M HCl.

$C_6H_{12}O_4N_2$ . Calculated. N 17.50.

Found. " 17.50.

*Preparation of Sarcosyl-Alanine.*—20 gm. of chloroacetyl-alanine were allowed to stand 24 hours at room temperature with 100 cc. of 31 per cent aqueous methylamine and then concentrated under diminished pressure to a thick syrup. The syrup was heated on the water bath with a large

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<sup>8</sup> We are indebted to Dr. Kellogg and Prof. Boldyreff of the Battle Creek Sanitarium for the intestinal juice which they generously furnished for the present use.

excess of absolute alcohol until crystallization took place. The yield was 16 gm. The material was recrystallized by dissolving it in an equal weight of hot water and boiling with a large excess of absolute alcohol until crystallization began. On cooling overnight a precipitate of 12 gm. of the dipeptide was obtained. It melted at 171–172°C. with decomposition. For analysis it was dried at 100°C. over sulfuric acid, under diminished pressure.

0.1000 gm. substance required (Kjeldahl) 12.62 cc. 0.1 M HCl.

$C_6H_{12}O_5N_2$ . Calculated. N 17.50.

Found. " 17.67.

*Preparation of Methylalanyl-Alanine.*—31.5 gm. of bromopropionyl-alanine were allowed to stand 24 hours with 100 cc. of 31 per cent aqueous methylamine and then concentrated under diminished pressure to a thick syrup. The syrup was heated on the water bath with about 250 cc. of hot absolute alcohol until crystallization took place. The yield was 16 gm. The material was purified by dissolving it in about 50 cc. of hot water and adding five parts of hot absolute alcohol. 14.5 gm. of recrystallized material were obtained. It melted at 235°C. with decomposition. For analysis it was dried at 100°C. over sulfuric acid, under diminished pressure.

0.1000 gm. substance (Kjeldahl) 11.50 cc. 0.1 M HCl.

$C_7H_{14}O_5N_2$ . Calculated. N 16.10.

Found. " 16.08.

*Preparation of Sarcosyl-Glycyl-Glycine.*—15 gm. of chloroacetyl-glycyl-glycine were allowed to stand 2 days at room temperature with 45 cc. of 31 per cent aqueous methylamine and then concentrated under diminished pressure to a thick syrup. The syrup was heated on the water bath with about 150 cc. of hot absolute alcohol until crystallization took place. The material was purified by dissolving it in five parts of hot water and adding an equal volume of hot absolute alcohol. 9 gm. of recrystallized material were obtained. It melted at 250–253° with decomposition. For analysis it was dried at 100°C. over sulfuric acid, under diminished pressure.

0.1000 gm. substance required (Kjeldahl) 14.70 cc. 0.1 M HCl.

$C_7H_{13}O_4N_2$ . Calculated. N 20.69.

Found. " 20.58.

*Preparation of Methylalanyl-Glycyl-Glycine.*—26.7 gm. of bromopropionyl-glycyl-glycine (0.1 mol) were allowed to stand 24 hours at room temperature with 82 cc. of 31 per cent aqueous methylamine and then concentrated under diminished pressure to a thick syrup. The syrup was heated on the water bath with about 250 cc. of absolute alcohol until crystallization took place. The yield was 18 gm. The material was purified by dissolving it in 50 cc. of hot water and adding about five parts of hot absolute alcohol. To obtain a good analysis a second recrystallization was necessary. 12.7 gm. of pure material were obtained. It melted at 252–253° with decomposition. For analysis it was dried at 100°C. over sulfuric acid, under diminished pressure.

0.1000 gm. substance required (Kjeldahl) 13.90 cc. 0.1 M HCl.

$C_8H_{15}O_4N_2$ . Calculated. N 19.35.

Found. " 19.46.

TABLE I.  
*Hydrolysis of Dipeptides With Erepsin at 32°C.*  
 Each solution was 1.0 molar and contained 2 cc. of enzyme solution (per 10 cc.).

Time.	GG <i>A</i> = 0.90			AG		GA		AA <i>A</i> = 0.33			SA		°AG		ApG	
	<i>V</i>		<i>ΔV</i>	<i>V</i>		<i>ΔV</i>	<i>V</i>		<i>ΔV</i>	<i>V</i>		<i>ΔV</i>	<i>V</i>		<i>ΔV</i>	
	cc.	cc.		cc.	cc.		cc.	cc.		cc.	cc.		cc.	cc.		
<i>hrs.</i>																
0	0.81			0.81		0.75		0.79		0.86		0.82	0.62		cc.	
0.25	0.83	0.02		0.83	0.02	0.77	0.02	(0.76)		0.88	0.02	0.83	0.01	0.62	0	
1	0.87	0.06	3.0	0.88	0.07	0.83	0.08	0.80	0.01	1.3	0.02	0.85	0.03	0.62	0	
2	0.90	0.09	2.3	0.94	0.13	0.86	0.11	0.81	0.02	1.3	0.08	0.87	0.05	0.62	0	
4				1.04	0.23	0.88	0.13	0.81	0.02	0.7						
7	1.19	0.38	1.4	1.19	0.38	0.94	0.19	0.84	0.05	1.0	0.10	1.01	0.19	0.63	0.01	
24	1.52	0.71	2.8	1.30	0.49	1.19	0.44	0.88	0.09	0.6	1.04	1.20	0.38	0.62	0	
29	1.54	0.73	2.5								1.05	1.21	0.39			
30				1.31	0.50	1.29	0.54	0.89	0.10	0.5						
48	1.63	0.82	1.4	1.30	0.49	1.40	0.65	0.94	0.15	0.5	1.10	1.24	0.42	0.63	0.01	
72	1.71	0.90				1.50	0.75	1.02	0.23	0.7		1.25	0.43	0.63	0.01	
Average.....			2.2													

TABLE II.  
Hydrolysis of  $\alpha$ AA and Glycyl Amide at  $41^{\circ}\text{C}$ .

Time.		With 2 cc. of enzyme solution.						No enzyme.	
		GG		$\alpha$ AA		GAm		GAm	
$t$	$1.5 t$	V	$\Delta V$	V	$\Delta V$	V	$\Delta V$	V	$\Delta V$
hrs.	hrs.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
0	0	0.81		0.63		0.79		0.77	
0.25	0.37	0.83	0.02	0.625	0.005	0.79		0.77	0
1	1.5	0.88	0.07	0.65	0.02	(0.83)		0.77	0
2	3	0.95	0.14	0.67	0.04	0.81	0.02	0.77	0
4	6	1.08	0.27	0.70	0.07	0.82	0.03	0.76	-0.01
7	10.5	1.25	0.44	0.75	0.12	0.83	0.04	0.77	0
24	36	1.62	0.81	0.90	0.27	0.93	0.14	0.77	0
48	72	1.74	0.93	1.00	0.37	1.04	0.25	0.77	0

Owing to the faster reaction rate resulting from higher temperature, the values of  $t$  are multiplied by 1.5 in order that the data of  $\alpha$ AA and GAm may be plotted in Fig. 1 and compared with the data in Table I.

TABLE III.  
Hydrolysis of Tripeptides With Ercpsin at  $25^{\circ}\text{C}$ .

Each solution was 0.75 molar with 2 cc. enzyme solution (per 10 cc.). Due to insolubility GGG was diluted to 15 cc.

Time.	GG				GGG				AGG			°AGG			
	$c = 0.75$ $A = 0.80$				$c = 0.50$ $A = 0.80$				$c = 0.75$			$c = 0.75$			
$t$	$V$	$\Delta V$	$\frac{\Delta V}{c}$	100k	$V$	$\Delta V$	$\frac{\Delta V}{c}$	100k	$V$	$\Delta V$	$\frac{\Delta V}{c}$	$V$	$\Delta V$	$\frac{\Delta V}{c}$	
hrs.	cc.	cc.			cc.	cc.			cc.	cc.		cc.	cc.		
0	0.60				0.40				0.60			0.60			
0.25	0.62	0.02	0.03	6.4					0.65	0.05	0.07	0.70	0.10	0.13	
0.50					0.50	0.10	0.20	31	0.68	0.08	0.11	0.72	0.12	0.16	
1.0	0.66	0.06	0.08	4.6	0.55	0.15	0.30	20	0.70	0.10	0.13	0.73	0.13	0.17	
2.0	0.70	0.10	0.13	3.9	0.68	0.28	0.56	26	0.76	0.16	0.21	0.74	0.14	0.19	
4.0	0.79	0.19	0.25	4.1	0.84	0.44	0.88		0.90	0.30	0.40	0.82	0.22	0.29	
7.0	0.83	0.25	0.31	4.4	0.95	0.55	1.10		0.98	0.38	0.51	0.87	0.27	0.36	
2.4	1.15	0.55	0.73	4.4	1.15	0.75	1.50		1.22	0.62	0.83	1.06	0.46	0.61	
Average.....				4.3					26						

*Preparation of Bromopropionyl-Sarcosine.*—21.6 gm. of sarcosine were dissolved in 240 cc. of sodium hydroxide (1 equivalent) and treated alternately with 50 gm. of bromopropionyl chloride (1.2 equivalents) and 396 cc. of normal sodium hydroxide, under cooling, each reagent being added in ten equal portions. The solution was then neutralized with 72 cc. of 5 normal hydrochloric acid and concentrated to dryness under diminished pressure. The residue was extracted several times with warm ether. The combined ether extracts (total 400 cc.) were dried over sodium sulfate and concentrated to a thick syrup. The syrup was stirred up with a little benzene. On cooling overnight under diminished pressure the syrup crystallized. It was filtered and washed with benzene. A yield of 18 to 27 gm. was obtained. A second crop was obtained by treating the filtrate with an excess of petroleic ether. The substance is very soluble in water and in alcohol, less soluble in chloroform, in ether, and in benzene, and insoluble in petroleic ether. It melted at 84°C. For analysis it was dried over sulfuric acid, under diminished pressure.

0.2000 gm. substance required (Kjeldahl) 8.80 cc. 0.1 M HCl.

$C_6H_{10}O_2NBr$ . Calculated. N 6.25.

Found. " 6.16.

*Preparation of Methylalanyl-Sarcosine Anhydride.*—20 gm. of bromopropionyl-sarcosine were allowed to stand 24 hours at room temperature with 80 cc. of 25 per cent aqueous methylamine and then concentrated under diminished pressure to a thick syrup. The syrup was extracted several times with hot benzene (total 300 cc.). The combined benzene extracts were concentrated under diminished pressure to a small volume and were then taken up with an excess of absolute ether. On cooling and stirring the methylalanyl-sarcosine anhydride crystallized. The yield was 5 gm. The substance is soluble in alcohol, in acetone, in chloroform, and in benzene and insoluble in ether and in petroleic ether. It gave no color reaction on boiling with fresh copper hydroxide. It melted at 78–80°C. For analysis it was dried over sulfuric acid, under diminished pressure.

0.1000 gm. substance required (Kjeldahl) 12.77 cc. 0.1 M HCl.

$C_7H_{12}O_2N$ . Calculated. N 17.94.

Found. " 17.88.

*Preparation of Bromoisovaleryl-Sarcosine.*—18 gm. of sarcosine were dissolved in 200 cc. of normal sodium hydroxide (1 equivalent) and treated alternately with 50 gm. of bromoisovaleryl chloride (1.2 equivalents) and 330 cc. of normal sodium hydroxide under cooling, each reagent being added in ten equal parts. The solution was then neutralized with 60 cc. of 5 normal hydrochloric acid. The bromoisovaleryl-sarcosine immediately precipitated as an oil which crystallized on cooling and stirring. The yield was 39 gm. The substance is soluble in ether, in chloroform, in alcohol, in acetone, and in benzene and insoluble in petroleic ether. It melted at 76–77°C. It was purified by dissolving it in a minimum amount of warm ether, and adding a large excess of petroleic ether.

0.2000 gm. substance required (Kjeldahl) 7.75 cc. 0.1 M HCl.

$C_8H_{14}O_3NBr$ . Calculated. N 5.56.

Found. " 5.56.

*Preparation of Methylvaleryl-Sarcosine Anhydride.*—15 gm. of bromoisovaleryl-sarcosine were allowed to stand 6 days at room temperature with 35 cc. of 25 per cent aqueous methylamine and then concentrated under diminished pressure to a thick syrup. The syrup was extracted several times with hot benzene. The combined benzene extracts (total 250 cc.) were concentrated under diminished pressure to a small volume and were then taken up in an excess of absolute ether. On cooling and stirring the methylvaleryl-sarcosine anhydride crystallized. The yield was 4 gm. The substance is soluble in alcohol, in benzene, in chloroform, and in acetone and insoluble in ether and in petroleic ether. It gave no color reaction on boiling with fresh copper hydroxide. It melted at 95°C. For analysis it was dried over sulfuric acid, under diminished pressure.

0.1000 gm. substance required (Kjeldahl) 10.68 cc. 0.1 M HCl.

$C_8H_{16}O_2N_2$ . Calculated. N 15.20.

Found " 14.95.

#### SUMMARY.

Continuing our study of the hydrolysis of peptides with erepsin, we find that seven other dipeptides give results which agree with our previous conclusions. They hydrolyze and form anhydrides at rates dependent upon the values of  $\left(\frac{K_a}{K_b}\right)^2$  involving the dissociation constants of the groups involved in the respective linkages. (Due to change in notation  $K_b$  is proportional to the reciprocal of  $K_b$  as used in previous papers in this series.)

These results were corroborated quantitatively in the case of alanyl-alanine (AA) and qualitatively for the other peptides. Methyl groups on the carbon atom (as in alanine) have the same effect as on the nitrogen (as in sarcosine) but to a smaller extent.

The values of the *dissociation* constants ( $K_a$  and  $K_b$ ) should be nearly proportional to the *intrinsic* constants ( $K_o^A$  and  $K_o^B$ ) of the respective groups, if we compare dipeptides with each other. However, in comparing dipeptides with tripeptides there should be considerable difference. We have hydrolyzed three tripeptides and find that the rates of hydrolysis are such as prove that the stabilities of the bonds are functions of the *intrinsic* constants

$\left(\frac{K_o^A}{K_o^B}\right)$  rather than of the dissociation constants. This is



theoretically to be expected. Tripeptides hydrolyze about ten times as fast as similar dipeptides. Higher peptides should be about the same as tripeptides, if we correct for ionization and change in enzyme activity with pH. A long chain of similar amino acids should be split by acid equally well at all the amide bonds (except the end two).

It is shown that erepsin fails to attack an anion (asparagylglycine, ApG, is not hydrolyzed), while neutral molecules (all simple peptides below  $pG_2$ ) and also cations (glycyl-amide) are hydrolyzed by erepsin above pH 7.

## A CRITICAL EVALUATION OF HAHN'S QUANTITATIVE METHOD FOR DETERMINING PROTEIN AND PROTEOSE.\*

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An accurately standardized method for determining quantitatively the content of whole protein and of proteose in a solution is much to be desired. Especially is this true when attempts are made, as with tuberculin, to ascertain whether the biological activity of a preparation is associated with the protein or with the smaller proteose molecules derived therefrom.

Such a method will, of necessity, employ as reagents, protein precipitants. Many studies have been made by previous investigators to select the precipitant which will differentiate most sharply between the whole protein molecule and its smaller fragments—the proteoses. Trichloroacetic acid is generally accepted to be the best reagent for this purpose. This acid was used in 1892 by Fränkel (1) to free glycogen of protein. Starling (2) found that it would not precipitate Grübler's commercial "peptone" but that it was efficient in separating the coagulable proteins in plasma, serum, and blood. Greenwald (3) employed trichloroacetic acid as a precipitant in the estimation of non-protein nitrogen in blood, and showed that when it was followed by kaolin treatment, the protein was precipitated completely. Amino acids are not adsorbed by the precipitate.

Hiller and Van Slyke (4) found that whole protein from blood is completely precipitated by trichloroacetic acid and that the precipitate does not contain free monoamino acids or peptide nitrogen. If it is desirable to precipitate, along with the protein, all

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of the decomposition products except amino acids, they recommend picric and tungstic acids as most efficient, and meta-phosphoric acid, colloidal iron, and mercuric chloride, as less efficient.

A quantitative comparison of the efficiency of phosphomolybdic, meta-phosphoric, and trichloroacetic acids, as precipitants, was made by Sjollesma and Hellerschy (5). Their results led them to conclude that trichloroacetic acid precipitated only the whole protein and that phosphomolybdic acid and meta-phosphoric acid precipitated a part of the decomposition products along with the protein.

With this information Hahn (6) designed a method for determining quantitatively the amount of protein decomposition products in the serum of patients suffering from different diseases. He precipitated the serum with 2.5 per cent trichloroacetic acid and determined the nitrogen in the filtrate. A similar amount of serum was precipitated with phosphotungstic acid and again the nitrogen determined in this filtrate. The difference between these two nitrogen figures represents the nitrogen in the protein decomposition products or proteose fraction. He determined the efficiency of the method by showing that casein which had been hydrolyzed for a short time gave a precipitate with phosphotungstic acid but none with trichloroacetic acid, and also that uric acid and creatinine were not precipitated by phosphotungstic acid. It is this method which has been critically examined in this investigation.

#### *Method.*

A 1 per cent protein suspension was prepared and duplicate 1 cc. samples were used for total nitrogen determinations. The solution was filtered through hardened filter paper and again 1 cc. samples were tested for total nitrogen; this figure being the soluble total nitrogen.

Two 2 cc. samples were precipitated with 8 cc. of 20 per cent trichloroacetic acid, making a 16 per cent concentration, and after standing 15 minutes the precipitate was filtered off through hardened filter paper. The filtrate was always clear. 1 cc. of concentrated  $\text{H}_2\text{SO}_4$  was added to 8 cc. of the filtrate in a large Pyrex test-tube, and the liquid evaporated over the free flame until it charred. Nitrogen was determined by the micro-Kjeldahl method, as outlined below.

Two more 2 cc. samples were precipitated with 8 cc. of 5 per cent phosphotungstic acid (containing 30 cc. of concentrated  $\text{H}_2\text{SO}_4$  per liter), and this precipitate was filtered off through hardened paper and 8 cc. of the filtrate evaporated with 1 cc. of concentrated  $\text{H}_2\text{SO}_4$  to the fuming stage and then digested as usual. This digestion requires constant attention. The mixture bumps badly because of the large amount of solid inorganic matter which is unavoidably present.

The nitrogen in the filtrate from 1 cc. of protein solution precipitated by phosphotungstic acid is the *residual nitrogen*, or the nitrogen of the monoamino acids and undetermined nitrogen. The nitrogen in the filtrate from 1 cc. of protein solution precipitated by trichloroacetic acid is the proteose plus residual nitrogen, and therefore, by difference between these two filtrate nitrogens, the *proteose nitrogen* is obtained. (This proteose nitrogen figure really includes diamino acids, since they have been shown by Van Slyke (7) to be precipitated by phosphotungstic acid. The error is so small, as illustrated below, that it can be disregarded in this work.) The difference between the nitrogen in the trichloroacetic acid filtrate and the total nitrogen is equal to the *whole protein nitrogen*. Blanks were subtracted in all cases.

A very important factor for consideration in this method is the concentration of trichloroacetic acid to be used. Hiller and Van Slyke (4) showed that the percentage of nitrogen in the filtrate from a trichloroacetic precipitation of Witte peptone varied with the amount of trichloroacetic acid used; for example, after precipitation with 2.5 per cent there was 85.4 per cent N in the filtrate; with 5 per cent, 77.9 per cent N; with 10 per cent, 62.5 per cent N. With the sample of Witte peptone used by the author, 16 per cent trichloroacetic acid was used and no more precipitate was produced by the addition of more acid. Only 52.2 per cent of the original nitrogen was found in the filtrate. It had been found on different occasions that 10 per cent acid is not sufficient to produce complete precipitation and so a minimum of 16 per cent was used as a routine and in all of the tests made this was found to be adequate. A 16 per cent concentration of trichloroacetic acid does not hydrolyze proteins under the conditions of these experiments. This is amply illustrated in the subsequent pages and can be inferred from the results of Greenwald,

who was able to show that a 22 per cent concentration was not hydrolytic.

In determining residual nitrogen, tungstic acid is recommended by Hiller and Van Slyke and should theoretically be a better precipitant than phosphotungstic acid, since it will not precipitate the diamino acids. The bumping which occurs with this reagent, however, is so much worse than when phosphotungstic acid is used, that a comparison of the determination with the two reagents was made. The residual nitrogen in crystalline ovalbumin, found by means of phosphotungstic acid, was 0.1 per cent N; by the tungstic acid, 0.085 per cent N. In two casein preparations the residual nitrogen determined by means of phosphotungstic acid precipitation was 0.18 per cent N and 0.14 per cent N; by tungstic acid, 0.18 per cent N and 0.15 per cent N respectively. The two methods checked so closely that it was deemed justifiable to use the former in the following analyses.

The nitrogen in the filtrates was determined by the micro-Kjeldahl method. The accuracy of the results was not affected by the presence of the trichloroacetic and phosphotungstic acid reagents, and, therefore, no attempt was made to remove them before continuing with the analyses. After the filtrates were concentrated with  $\text{H}_2\text{SO}_4$  to the fuming stage, solid  $\text{Na}_2\text{SO}_4$  (about 1 gm.) and a small piece of  $\text{CuSO}_4$  were added and they were digested for  $\frac{1}{2}$  hour after they were colorless. After cooling, about 5 cc. of distilled water were added. 6 cc. of a saturated solution of NaOH were introduced through a pipette, after the distillation apparatus (Koch's modification of Folin's apparatus) was closed. The ammonia was distilled over into  $\text{N}/100$  HCl and the distillate titrated with alizarin and  $\text{N}/100$  NaOH. Duplicate samples were run in all cases and no results were accepted which varied more than 0.2 cc.  $\text{N}/100$  alkali, making an error of about 1 per cent or less.

### *Results.*

Determinations of the percentage of whole protein, proteose, and residual nitrogen were made on a variety of highly purified proteins and when possible upon crystallized proteins. In order to determine the applicability of the method to proteins in general, representatives of four different protein groups were chosen;

crystallized egg albumin (albumin), crystallized edestin (globulin), gliadin (prolamin), two samples of casein (phosphoprotein), and Witte peptone and a proteose fraction of the latter. Table I shows that all of the whole proteins, which had been purified by the best methods known at the present time, are precipitated quantitatively (within at most 2 per cent) by trichloroacetic acid, under the conditions specified. From these data, the conclusions can therefore be drawn that trichloroacetic acid precipitates pure proteins practically quantitatively, and this supports Greenwald's findings for the proteins of the blood.

TABLE I.

Substance tested.	Total N.		
	As protein.	As proteose.	As residual.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Albumin.			
Crystalline Ovalbumin 1.....	99.19	0.00	0.08
"                    "      2.....	98.32	0.83	0.83
Impure Ovalbumin 1.....	86.15	13.13	0.71
"                    "      2.....	68.80	16.89	14.30
Globulin.			
Edestin, crystalline.....	98.73	0.63	0.63
Prolamin.			
Gliadin (Vickery).....	97.80	0.73	1.47
Phosphoprotein.			
Casein (author).....	98.61	0.00	1.46
"      (Osborne).....	98.24	0.68	1.06
Witte peptone.....	47.63	44.55	7.81
Proteose.....	0.00	75.28	24.79

On the other hand, by this method, impure proteins are shown not to contain 100 per cent whole protein. Two samples of egg protein, for example, which correspond to the fraction precipitated by the addition of more  $(\text{NH}_4)_2\text{SO}_4$  after the crystalline ovalbumin is removed (or chiefly, the conalbumin fraction and some proteose) are seen to contain comparatively less whole protein (68.80 per cent and 86.15 per cent) and much more proteose (16.89 per cent and 13.13 per cent) than the crystalline protein itself. And as might be expected, even more proteose (44.55 per cent) is found in Witte peptone.

If we can assume, therefore, that crystalline proteins and proteins prepared by repeated solution and reprecipitation and dialysis consist of only whole protein molecules, and if trichloroacetic acid precipitates them practically quantitatively while it will precipitate only the whole protein from admittedly impure protein fractions or from Witte peptone, then trichloroacetic acid can be used as a reagent for separating whole protein from proteose.

The one further question to be answered before the absolute accuracy of such a method can be determined, is whether this reagent will be as efficient in precipitating whole protein when proteose is also present. An experiment was therefore designed to test this point. Proteose was purified by discarding from Witte

TABLE II.

Substance tested.	Total N.	Total N.		
		As protein.	As proteose.	As residual.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Crystalline ovalbumin.....	13.18	98.33	0.83	0.83
Proteose.....	13.31	0.00	75.28	24.79
Mixture of above.....	13.24	52.79	34.82	12.38
“ (by calculation).....	13.24	48.94	38.21	12.83
Difference.....	0.00	+3.85	-3.39	-0.45

peptone all that would precipitate with trichloroacetic acid, dialyzing the clear filtrate containing only the proteose and residual nitrogen and drying the non-dialyzable residue. Nitrogen partition analyses were run upon (1) the crystallized ovalbumin alone, (2) on the proteose alone, and (3) on a mixture of equal parts ovalbumin and proteose. The results are contained in Table II. A comparison of the results obtained on the mixture, with the theoretical, show that when the protein and proteose were mixed, 3.85 per cent more nitrogen was found in the whole protein fraction than there should be and on the other hand, 3.39 per cent less of the nitrogen as proteose and 0.45 per cent less of the nitrogen as residual was found in the mixture. This is interpreted to mean that in the concentrations used, 3.39 per cent of the proteose and 0.45 per cent of the residual nitrogen were carried down from solutions of mixtures of protein and proteose with the trichloro-

acetic acid precipitate and were then erroneously considered as whole protein. So that the method here outlined is accurate within about 3.5 to 4.0 per cent.

Furthermore, attention should be called to the fact that this degree of accuracy will be obtained only when the concentration of protein solution here recommended (namely, 0.01 gm. protein per cc.) is used. Determinations were run upon the same protein (impure egg albumin) when it existed in concentrations of 0.1 gm. per cc. and 0.01 gm. per cc. There appeared to be 4.5 per cent more whole protein nitrogen and 3 per cent more proteose nitrogen precipitated from the more concentrated solution than from the dilute one, and a corresponding loss in the residual nitrogen. The precipitate in the more concentrated solution was very thick and presumably occluded considerable residual nitrogen, and proteose nitrogen.

Concentration of impure egg albumin. gm. per cc.	Protein N. per cent	Proteose N. per cent	Residual N. per cent
0.01	68.8	16.9	14.3
0.1	73.3	19.9	6.8

#### SUMMARY.

Hahn's method, with modifications as described, is reliable with an experimental error of no more than 1 per cent for determining the percentage of whole protein, proteose, and residual nitrogen.

Highly purified and when possible crystalline representatives of different protein groups were quantitatively precipitated, to within 1 to 2 per cent, by trichloroacetic acid. This finding supports the conclusions of Greenwald and others with blood proteins. Impure ovalbumin preparations were shown to contain only 86.15 per cent and 68.8 per cent and a sample of Witte peptone only 47.63 per cent whole protein nitrogen by this method.

When equal parts of a purified protein and a pure proteose are mixed, trichloroacetic acid precipitates the whole protein quantitatively, but in addition, carries down with the precipitate some of the proteose and residual nitrogen, which then is erroneously considered as whole protein. Within this limit the method is accurate. In the experiment here described 3.85 per cent of the



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proteose and residual nitrogen was included in the whole protein fraction.

A considerable error is introduced when a protein solution ten times as concentrated as that recommended (1 per cent) is used, because of occlusion of the decomposition products with the whole protein precipitate.

Sincere appreciation is expressed to Dr. Milton T. Hanke for his many helpful suggestions and criticisms.

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# SOME NITROGENOUS CONSTITUENTS OF THE CAULIFLOWER BUD.

## I. PROTEIN FRACTIONS.\*

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The fact that plants are the ultimate source of physiologically available nitrogen for animals has enhanced the value of investigation of plant proteins. The storage proteins from seeds were the first to be extensively studied because they were comparatively easy to obtain in fair degree of purity and in quantity. More recently, attention has been turned to the proteins in leaves; and spinach (1-3), cabbage (2), scarlet runner bean (2), alfalfa (4, 5), maize (*Zea mays*) (6), and several other leaves (7) have been investigated. Attention has been redirected to the non-protein nitrogenous compounds in the aqueous extract of leaf pulp by the recent studies of Vickery (8-13) which promise to yield much of interest to the student of nutrition.

In the present investigation the proteins of the bud—a type of tissue which has hitherto received little, if any, attention—have been studied. The material used was the cauliflower (*Brassica oleracea*, var. *botrytis* L.), the edible head of which is considered to be a malformed and condensed flower stem and buds of flower clusters.

### *Distribution of Nitrogen in the Edible Portion of the Cauliflower.*

Four lots of cauliflower each of twenty-four heads were purchased in January, February, September, and October, 1925. With slight modifications, each was treated in the same manner.

\* The experimental data in this paper are taken from the dissertation submitted by Mary C. McKee in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1926.

† Phi Mu Fellow of the American Association of University Women, 1925-1926.

The outer leaves were removed, the heads broken apart, and the edible portions of firm white heads were selected for analysis. The material was ground several times in an electric meat grinder, using successively finer plates. Samples were removed for determination of total nitrogen, dry solids, and ash. The pulp was placed in heavy muslin bags and pressed in a Buchner press at 350 to 400 kilos per sq. cm. (2). The milky juice was filtered with suction through paper pulp which removed all visible solid particles but did not clarify the solution. Distilled water was added to the pulp which was again pressed, the extract filtered, added to the original juice, and heated to the boiling point. At about 60°C., a coagulum formed, the solution cleared, and became reddish brown in color. After cooling, the coagulum was removed by centrifuging.

The residual pulp was extracted in the same way five or six times more, each time using about 4 liters of water. These extracts were united and treated as before. Very little coagulable protein was obtained from them although soluble nitrogen was removed up to the fifth or sixth extraction. Except during the time that the pulp was being pressed, it was kept in the refrigerator at 35–40°C.

The pulp was next treated with 0.3 per cent sodium hydroxide solution. The extraction was repeated five or more times and over 20 liters of solvent were used. The solution was brought with acetic acid to a hydrogen ion concentration of approximately 5.4, and a precipitate, obtained at this point, was centrifuged off.

The filtrates from the above extractions were concentrated separately by evaporation at 80–85°C. in a current of air. These were preserved with toluene and kept for future study.

*Analysis.*—The data obtained from the analysis of the cauliflower purchased in October, 1925, are given in Table I. The analyses of the three earlier lots of cauliflower have been averaged and are given in Table II.

The data recorded in Tables I and II show that the edible portion of the cauliflower plant, as purchased at different times during the autumn and winter, yielded protein fractions, designated as "coagulum" and "precipitate," containing less than 15 per cent of the total bud nitrogen. They show, also, that the greater part of the nitrogen was present in compounds which are soluble in water or in dilute saline solution while a much smaller amount occurred in substances insoluble in these solvents but soluble in dilute alkalies.

From 14 to 17 per cent of the total nitrogen remained in the pulp after repeated extraction with water and alkali. If a way can be found to grind the material more thoroughly or to break

down the cell walls by some other method, it is possible that more of the residual nitrogen may become extractable by water or dilute alkali.

TABLE I.

*Distribution of Nitrogen in Cauliflower, Purchased October, 1925.*

Weight of cauliflower pulp, 9220 gm.

	Dry solids.	Dry solids in terms of total dry solids.		Nitrogen in terms of total nitrogen.		Ash in terms of total ash.	Ash in terms of total dry solids.
		gm.	per cent	gm.	per cent		
Whole cauliflower . . . . .	696	100.0	40.4	100.0	64.7	100.0	9.3
Expressed juice . . . . .	160	23.0	13.5	33.5	25.5	39.4	3.7
Aqueous extracts . . . . .	195	28.1	15.0	37.3	33.6	52.2	4.9
Coagulum . . . . .	33.3	4.8	4.6	11.3	0.7	1.1	0.1
Pulp, after aqueous extraction . . . . .	302	43.4	11.1	27.5	8.0	12.3	1.2
Sodium hydroxide extracts . . . . .			4.9	12.2			
Precipitate . . . . .	11.5	1.7	1.5	3.1	0.2	0.3	0.02
Pulp, after extraction with water and NaOH . . . . .	256		5.8	14.1			

TABLE II.

*Average Values Obtained from Three Lots of Cauliflower Purchased at Different Times of the Year, Each Lot Containing 2½ Heads.*

	Dry solids in terms of total dry solids.	Nitrogen in terms of total nitrogen.	Ash in terms of total ash.	Ash in terms of total dry solids.
	per cent	per cent	per cent	per cent
Whole cauliflower . . . . .	100.0	100.0	100.0	10.4
Expressed juice } . . . . .	57.9	67.3	88.8	9.0
Aqueous extracts }				
Coagulum (two lots only) . . . . .	3.6	8.5	0.4	0.1
Pulp, after aqueous extraction . . . . .	38.2	29.3	12.1	1.3
Sodium hydroxide extracts . . . . .		11.6		
Precipitate (two lots only) . . . . .	1.6	3.5	0.5	0.03
Pulp, after extraction with water and alkali . . . . .		16.9*		

*Partial Analysis of Expressed Juice and Aqueous Extracts of Cauliflower.*

This material consisted of the combined expressed juice and aqueous extracts of the pulp of the cauliflower purchased in

October, 1925, and contained 70.7 per cent of the entire nitrogen of the edible portion of this sample. After the removal of the coagulum and concentration, the filtrate contained 55.2 per cent of the cauliflower nitrogen.

Aliquot samples were analyzed for ammonia before and after hydrolysis for 30 hours with 20 per cent hydrochloric acid. The value given for amide nitrogen is taken as the increase in ammonia after this hydrolysis. Experiments in which the milder hydrolysis recommended by Sachsse (14) was employed indicated very little difference between the results of the two methods. The amino

TABLE III.  
*Nitrogenous Constituents of the Coagulum-Free Aqueous Extracts of Cauliflower Pulp.*

Form of nitrogen.	Nitrogen in filtrate.	Nitrogen in cauliflower.
	<i>per cent</i>	<i>per cent</i>
Unhydrolyzed filtrate.		
Total N. . . . .	100 0	55.2
Ammonia N. . . . .	15.1	8 4
Amino N. . . . .	34 2	18.9
Other N. . . . .	50 7	27 9
Hydrolyzed filtrate.		
Total N. . . . .	100.0	55.2
Ammonia N. (as recorded above). . .	15 1	8 4
Amide N. . . . .	9 6	5.3
Amino N. . . . .	61.3	33 8
Other N. . . . .	14.0	7 8

nitrogen was determined by the use of the Van Slyke micro apparatus and the results are recorded in Table III.

It is of interest to note the large amount of free amino nitrogen in the aqueous extract of the cauliflower pulp and to compare it with the observations of Vickery (8) on the protein-free filtrate obtained from the juice pressed from fresh alfalfa leaves. His material contained 4 per cent of ammonia nitrogen, 8.3 per cent of amide nitrogen, and 36.5 per cent of free amino nitrogen. Earlier work of Osborne, Wakeman, and Leavenworth (15) showed that after hydrolysis the protein-free alfalfa filtrate gave ammonia nitrogen values more than three times and amino nitrogen values practically twice as great as the unhydrolyzed material.

*Coagulum.*

*Preparation and Purification.*—The coagulum was washed repeatedly with boiling water until the washings no longer gave a positive Molisch test. It was then treated with ethyl alcohol solutions of increasing concentrations and finally with ether. After removal of ether by filtration the coagulum was dried. It was found to represent about 4.8 per cent of the weight of the dry solids of the cauliflower pulp used. It was light brown in color, insoluble, or only very slightly soluble, in water, alcohol, ether, dilute acids, or alkalies, but soluble after long heating in a large excess of boiling 60 per cent alcohol containing 0.3 per cent sodium hydroxide. The dried powder gave positive biuret, xanthoproteic, Millon, and Hopkins-Cole color tests. It also gave a strongly positive Molisch test, and the hydrolyzed material showed a reducing action on copper salts. The monosaccharides produced by hydrolysis were not fermented by yeast; they gave the orcinol test characteristic of pentoses.

*Analysis.*—The moisture-free material contained nitrogen, 13.7 per cent; ash, 2.1 per cent; sulfur, 0.94 per cent; phosphorus, 0.52 per cent. The nitrogen estimated on the ash- and moisture-free material was 14.1 per cent. The determination of sulfur was made by the magnesium nitrate method and the phosphorus was estimated in the filtrate after the removal of the sulfur.<sup>1</sup> The high ash and comparatively low nitrogen content indicate that the protein has not been isolated in pure form.

In an attempt to free the product from substances other than protein, a small amount of the moist coagulum was boiled under a reflux condenser for 12 hours with 60 per cent alcohol containing 0.3 per cent sodium hydroxide. The greater part of the coagulum dissolved. The solution was filtered and acidified with dilute acetic acid; the precipitate so obtained was washed repeatedly with boiling alcohol, then with ether, and finally dried to constant weight at 107–110°C. It was found to contain 14.5 per cent of nitrogen (with no correction for ash) but still gave a positive Molisch test.

The reducing substance obtainable from the coagulum was

<sup>1</sup> Official and tentative methods of analysis, *Assn. Off. Agric. Chem. Washington*, 1925.

determined by the Shaffer and Hartmann (16) micro method. The sample, heated for 6 hours with 50 cc of 3 per cent hydrochloric acid in a boiling water bath, yielded 4.1 per cent of reducing material expressed as glucose, in the ash- and moisture-free material.

*Amino Acid Content of the Coagulum.*—An analysis of the coagulum was made by the method of Van Slyke (11, 18) hydrolyzing for 30 hours. The phosphotungstates were decomposed by the amyl alcohol-ether method. The arginine was determined by the

TABLE IV.

*Distribution of Nitrogen Obtained by the Van Slyke Method in Coagulum.*

	Nitrogen.
	<i>per cent</i>
Amide N. ....	7.03
Humin N. ....	3.88
Basic N. ....	26.30
Cystine N. ....	0.70
Arginine N. ....	12.28
Histidine N. ....	2.68
Lysine N. ....	10.66
Amino N, filtrate. ....	55.85
Non-amino N, filtrate. ....	3.60
<hr/>	
Total N recovered. ....	96.68
<hr/>	
	Protein.
	<i>per cent</i>
Arginine. ....	4.87
Histidine. ....	1.30
Lysine. ....	7.07

Nitrogen figures corrected for solubility of phosphotungstates.

Plimmer (19) modification of the Van Slyke method. So much humin was formed during the hydrolysis that filtration was necessary before samples were taken for the determination of total nitrogen. The value for the total nitrogen of the hydrolyzed material as used in compiling Table IV is, therefore, the sum of the humin nitrogen, removed from the acid solution, and the nitrogen remaining in the filtrate.

In running Kjeldahl nitrogen determinations on the filtrate from the bases, there was much annoyance caused by excessive bumping during the digestion until 5 gm. of anhydrous sodium sulfate were added to each flask. This retained the precipitate in solution and the oxidation ran smoothly to completion.

The average results of duplicate determinations made upon the coagulum are given in Table IV.

Analysis of the coagulum by the method of Folin and Looney (20) showed tyrosine 5.76 per cent and cystine 2.35 per cent.<sup>2</sup> Tryptophane, determined by the method of May and Rose (21), was 0.52 per cent.

While it is realized that the preparation used was not a pure protein and may have contained foreign material which interfered with the color reactions, the results are recorded with the thought that they may be at least indicative of the composition of the coagulum.

### *Precipitate.*

*Preparation and Purification.*—The precipitate was washed with cold water, dissolved in 0.3 per cent sodium hydroxide solution, filtered through paper pulp with suction, and reprecipitated with acetic acid. This process was repeated twice. The solid was thoroughly washed with cold water many times and then with alcohol of increasing concentration. The first alcoholic extracts were red in color and the washing was continued until they were practically colorless. The precipitate was then treated with ether, allowed to stand in ether for 24 hours, filtered, and dried. The dried powder was light brown in color, insoluble in water and saline solutions, but soluble in dilute alkalis. An alkaline solution gave strongly positive biuret, xanthoproteic, Millon, and Hopkins-Cole tests. On acidification of the alkaline solution with hydrochloric acid, the protein precipitated but was soluble in strong excess. The preparation gave a strong Molisch test.

The yield amounted to 1.7 per cent of the dry solids of the cauliflower pulp; i.e., the quantity of precipitate is about one-third that of the coagulum.

*Analysis.*—The moisture-free material contained nitrogen, 13.4

<sup>2</sup> For the amino acids used as standards we are indebted to the Laboratory of the Connecticut Agricultural Experiment Station.



per cent; ash, 1.71 per cent; sulfur, 0.83 per cent; and phosphorus, 0.21 per cent. The nitrogen of the estimated ash- and moisture-free material was 13.60 per cent. The precipitate yielded 6.91 per cent of reducing substance calculated as glucose.

*Amino Acid Content of the Precipitate.*—Due to the scarcity of material, the determination of the distribution of amino acid groups by the Van Slyke method was not made in duplicate but

TABLE V.  
*Distribution of Nitrogen Obtained by the Van Slyke Method in Precipitate.*

	Nitrogen.	
	Lot 1.	Lot 2.
	<i>per cent</i>	<i>per cent</i>
Amide N .....	6.98	6.97
Humin N .....	3.69	3.64
Basic N .....	27.97	31.74
Cystine N .....	1.06	1.03
Arginine N .....	12.03	13.98
Histidine N .....	4.42	6.11
Lysine N .....	10.46	10.62
Amino N, filtrate .....	50.12	48.89
Non-amino N, filtrate .....	7.79	8.30
Total N recovered .....	96.55	99.54
	Protein.	
	Lot 1.	Lot 2.
	<i>per cent</i>	<i>per cent</i>
Arginine .....	5.02	5.87
Histidine .....	2.19	3.06
Lysine .....	7.41	7.53

Nitrogen figures corrected for solubility of phosphotungstates.

instead one determination each was made upon the precipitates obtained from the cauliflower lots purchased in September and October, 1925, respectively. The precipitate obtained from the first lot contained 13.5 per cent of nitrogen; the second, 13.6 per cent. The results of these analyses are given in Table V.

The colorimetric determinations carried out as already described gave the following results: tyrosine, 7.6 per cent; cystine, 6.3

per cent; and tryptophane, 0.8 per cent. The unexpectedly high value for cystine is not supported by the total sulfur content of the precipitate. Evidently the colorimetric method employed is not applicable to this relatively impure material.

#### DISCUSSION.

The foregoing details regarding the distribution of the nitrogenous compounds in the bud, or edible portion, of the cauliflower indicate that only a small fraction of the nitrogen exists in the form of protein soluble in water, and still less as protein soluble in weak alkali. These two protein fractions, representing approximately 14 per cent of the total nitrogen of the material studied, together with the 14 per cent of nitrogen which remained, unextracted and unidentified, in the washed pulp, accounted for less than one-third, at most, of all the nitrogen present in the cauliflower bud. In other words, two-thirds of the nitrogenous constituents of the latter existed in forms as yet unseparated and unidentified.

The primary aim of the present work has been the isolation and purification of proteins. Carbohydrate was present in each of the preparations. This may occur either combined with the protein or it may be the contaminating part of a mixture. The difficulty experienced in removing this carbohydrate indicates little concerning its true relation to the protein.

Various methods have been proposed for the extraction of proteins from physiologically active plant tissue. Chibnall (22) treated leaf tissue with ether thereby plasmolyzing the cells and facilitating the removal of the vacuole fluid by means of pressure. The leaves were then thoroughly ground and extracted with water. This extract on treatment with the required amount of acid yielded a precipitate which consisted almost wholly of protein. By this means he distinguished between protein derived from the cytoplasm and that dissolved in the vacuole fluid. Osborne and his associates ground leaf tissue without previous treatment, extracted with water, and precipitated the proteins by means of alcohol. The separation of the protein from the extract by means of heat coagulation has been employed by Chibnall and Schryver.

The Chibnall method of plasmolysis proved to be of no assistance in breaking down the tougher cauliflower tissue; consequently, the

Osborne method of grinding, pressing, and extracting was employed in the present work. By this manipulation a thick milky juice was expressed. Filters assumed to be free from the danger of adsorbing the protein proved ineffective in clarifying the solution. It is likely that contaminating substances accompanied the protein as it was thrown out of solution during the coagulation. Repeated washing with boiling water and alcohol served to remove some, but not all, of the carbohydrate and mineral matter from the protein coagulum and apparently left other unidentified impurities untouched.

The attempt to isolate an undenatured protein from the alkaline extract of the pulp was even more disappointing. It is probable that the boiling hot reagents used in the purification of the coagulum removed impurities which in the absence of such treatment remained in the precipitate.

The purest protein preparations obtained from the cauliflower bud contained 14.1 and 13.6 per cent of nitrogen in the coagulum and precipitate respectively. For purposes of comparison, it is necessary to turn to the only other type of active protoplasmic tissue which has been studied hitherto, the leaf. Chibnall and Schryver (2) have worked with the cabbage, the scarlet runner bean, and spinach and obtained from them proteins which had a nitrogen content of 12.8 per cent, 13.3 per cent, and 12.6 per cent respectively. These figures are based on the moisture-free material.

In 1924, Chibnall and Nolan (5) isolated from the alfalfa leaf a protein preparation that contained 13.2 per cent of nitrogen and from maize leaves one which contained 14.4 per cent. Chibnall and Grover (7) have also isolated preparations from the leaves of cabbage, horse radish, New Zealand spinach, sun flower, and the fig, which contain 14.65, 14.4, 14.5, 13.7, and 13.4 per cent of nitrogen respectively.

In contrast with these low nitrogen values are the higher figures for preparations obtained from the spinach leaf by Osborne and Wakeman (1) and from the alfalfa by Osborne, Wakeman, and Leavenworth (4). Both of these contained about 15.5 per cent of nitrogen. Chibnall and Nolan (5) plasmolyzed the alfalfa leaf and separated a preparation of cytoplasmic protein which had a nitrogen content of 15.75 per cent. Chibnall (3) isolated

"spinacin," a protein from the spinach, which contained 16.25 per cent of nitrogen and was free from carbohydrates. Very recently, Chibnall and Grover (7) have reported the separation from the broad bean leaf (*Vicia faba*) of a protein containing 16.77 per cent of nitrogen. This is the highest content of nitrogen reported in any preparation of protein from actively metabolizing plant tissue.

The study of the distribution of the amino acid groups in the cauliflower proteins discloses an interesting similarity between them and the proteins isolated from the leaves. For comparison,

TABLE VI.  
*Distribution of Nitrogen as Obtained by the Van Slyke Method.*

	Alfalfa protein (Chibnall and Nolan).	Spinacin (Chibnall).	Cauliflower proteins.	
			Coagulum (McKee and Smith).	Precipitate (McKee and Smith).
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Amide N.....	5.51	6.93	7.03	6.97
Humin N.....	2.72	2.47	3.88	3.64
Cystine N.....	0.04	1.27	0.70	1.03
Arginine N.....	15.32	13.83	12.28	13.98
Histidine N.....	3.09	3.89	2.68	6.11
Lysine N.....	9.97	9.62	10.66	10.62
Amino N, filtrate.....	58.56	58.09	55.85	48.89
Non-amino N, filtrate.....	3.19	2.58	3.60	8.30
Total N recovered.....	99.20	98.66	96.68	99.54

the figures obtained by Chibnall and Nolan for the cytoplasmic protein isolated from the alfalfa and by Chibnall for spinacin are given in Table VI, together with those for the cauliflower proteins.

#### SUMMARY.

Analysis of the edible portion of the cauliflower shows that approximately 68 per cent of the nitrogen of this part of the plant belongs to constituents soluble in water or dilute salt solution; 12 per cent to compounds insoluble in water but soluble in dilute alkali; and 16 per cent to substances insoluble in both water and dilute alkali.

A further fractionation of the combined expressed juice and

aqueous extract showed that it contained about 8 per cent of the total bud nitrogen as ammonia nitrogen; 19 per cent as free amino nitrogen; 5 per cent as amide nitrogen; and 11 per cent as nitrogen in actually isolated protein preparations.

Dilute sodium hydroxide solution extracted about 11 per cent of the total nitrogen of the bud; 3 per cent of the total cauliflower nitrogen was subsequently separated as a protein preparation.

Two preparations rich in nitrogen and giving the protein color reactions have been isolated. The distribution of the nitrogen has been ascertained by the Van Slyke method. In both products, however, the percentage of nitrogen was lower than that usually found in pure proteins; both contained carbohydrates and perhaps other organic material.

The authors wish to acknowledge their indebtedness to Professor Lafayette B. Mendel and Dr. H. B. Vickery for advice and suggestions during the investigation.

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## ON FAT AND GLYCOGEN IN THE TISSUES IN EXPERIMENTALLY INDUCED OBESITY IN THE RAT.\*

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The remarkable obesity and sex infantilism which develop in rats following certain injuries to the brain in the neighborhood of the hypophysis have been described by Smith (1). The accumulation of fat proceeds very rapidly following the operation and the animals present a highly interesting material for the study of certain phases of fat metabolism. Some analyses of the fat in the livers and in the carcasses of these animals have been made with the hope of obtaining an indication as to whether the rapid accumulation of fat is due to some peculiarity in the fat itself rendering its utilization difficult, or whether the obesity is due to some other metabolic disturbance. In some cases the livers were also analyzed for glycogen.

This material was made available for study through the courtesy of Dr. P. E. Smith of the Department of Anatomy. The work was unavoidably interrupted before completion.

The results are presented in Table I. Some of these figures are perhaps the highest on record for the fat content of the animal body. There appears to be no striking qualitative difference in respect to degree of unsaturation between the body fat of the operated animals and that of their controls. The livers of the operated rats contain abnormally large amounts of fat with iodine values approximating those of the general body fat. The glycogen figures are of some interest in that in every case but one there was much more glycogen in the livers of the operated animals than in those of their controls. This seems to be an exception to Rosenfeld's (2) generalization that when the liver fat is high the glycogen is low and *vice versa*.

\* Aided by a grant from the Board of Research, University of California.

In the case of Animal 720, a separation of solid and liquid fatty acids from a sample of pure adipose tissue from the mesenteries was made with the following result (3).

Total fatty acid plus non-saponifiable, 18.5 gm.

Non-saponifiable, 1.0 per cent.

Iodine No. of fatty acids, 64.

Solid fatty acids, 29 per cent, iodine No. 3.

Liquid fatty acids, 67 per cent, iodine No. 93.

TABLE I.

	Operated, fat.								Normal controls.							
Number .....	103	339	56	525	522	551	444	720	104	285	340	524	521	549	442	55
Body weight, gm.....	539	248	790	276	333	220	364	372	238	223	201	189	188	210	267	300
Water in carcass, per cent.	29	47	26	44	29				62	64	60	65	62			62
Fat in carcass, per cent.....	58	24	65	44		25		73*	16	7.8	13	9.9	7	13		13.2
Iodine No. of fat in carcass.	59		72			76		64	63		74	77	72	89		74
Liver fat, per cent .....	24		9	18	10	5.1	6.6		3.6		5.6		3.1	3.7	4.2	5
Iodine No. of liver fat.....	65			71	76	77	68		106		92		97	82	92	90
Liver glycogen, per cent.....	0			3.6	4.3	3.6	4.4		1.2			0.83	1.8	1.7	1.0	

\* The average of triplicate analyses (81, 71, 68 per cent fat respectively). The hashed carcass "swimming" in oil was not easy to sample fairly. The lower figure is perhaps more nearly correct.

### Methods.

The carcasses, after removal of thyroids, adrenals, gonads, and livers at autopsy, were passed through a meat grinder. The hash was analyzed in duplicate or triplicate. Total fatty acids were determined by the saponification method as used by Leathes. Separation of solid and liquid fatty acids was effected as described by Sperry and Bloor (4). Iodine numbers were obtained by the Hanus method. Glycogen was determined according to Pflüger, titrating the glucose by the method of Shaffer and Hartmann. In Table I the figures for moisture, fat, and glycogen are expressed as per cent of the fresh tissue.

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## AMMONIA AND BLOOD SUGAR.

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In 1919 Fosse (6) showed that urea is formed by the oxidation of solutions containing such amounts of glucose and ammonia as are present in the blood. Recently Hynd (9) found that "A 2 per cent solution of glucose containing approximately 60 mg.  $\text{NH}_3$  showed no optical change at room temperature, but after 24 hours at  $50^\circ$  the rotation fell from  $+0.87^\circ$  to  $+0.29^\circ$  . . . . Similar results were obtained when ammonia was added to an aqueous solution of glucose dissolved in an aqueous phosphate mixture of  $\text{pH} = 7.40$ ."

These suggestive findings led us to study the effect of ammonia on blood sugar *in vivo* as well as the effect of glucose on blood urea *in vitro*.

### *Procedure.*

Rabbits of 2.3 to 2.6 kilos body weight, fasted for 24 hours (except in one case, in which the animal was fasted for 48 hours), were used for this purpose. During the hours of the experiment the rabbits were kept quiet in a tray but without restraint and there was no struggle when blood was taken from the marginal ear vein. In order to obtain the first portion of blood a small incision was made, from which it was also possible to obtain the later samples of one experiment. The bleeding was stopped each time by sticking a small piece of cotton on the wound. The samples consisted of 2 cc. of blood.

In one case six consecutive blood portions were taken in a period of  $2\frac{1}{2}$  hours from a normal rabbit in order to investigate the effect of bleeding on the blood sugar.

The blood was taken before and after subcutaneous injection of the following solution.

$\text{NH}_4\text{HCO}_3$  . . . . 32.5 gm.  
Normal saline to 1000 cc.

This mixture contains 7 mg. of  $\text{NH}_3$  in 1 cc.; i.e., the same amount as was used in the experiments of Biedl and Winterberg (3) with ammonia injections.

In order to determine the toxic dose, this solution in large doses was injected subcutaneously into two rabbits.

In three cases a urease solution (Arlco urease, diluted 1:100) was injected subcutaneously in order to raise the ammonia level in the blood, as was shown to be possible by Carnot and Gerard (4).

TABLE I.

*Effect of Subcutaneous Injections of Ammonium Carbonate Solution Containing 7 Mg. of  $\text{NH}_3$  per Cc.*

Rabbit No.	Time.	Ammonium carbonate solution.	Observations.
		cc.	
X	10.00 a.m.	20	No symptoms.
	3.00 p.m.	20	" "
	4.00 "	20	Convulsions.
	4.30 "		Died.
XI	9.00 a.m.	20	No symptoms.
	2.00 p.m.	20	" "
	3.00 "	20	Convulsions.
	3.45 "		Died.

The blood sugar was determined by the method of Folin and Wu.

In order to study the effect of glucose on blood urea nitrogen, glucose, free of nitrogen, was added to rabbit blood filtrates with a known urea nitrogen content, and subsequently the urea nitrogen was determined by the aeration method.

### Results.

The results of all experiments are presented in Tables I, II, III, and IV.

It can be seen from Table II that bleeding did not produce a great change in the blood sugar. Subcutaneous injections of

TABLE II.

*Effect of Subcutaneous Injections of Ammonium Carbonate Solution Containing 7 Mg. of  $NH_3$  in 1 Cc. on the Blood Sugar of Fasted Rabbits.*

Date.	Rabbit No.	Ammonium carbonate solution.	Time of injection.	Time when blood was taken.	Glucose.
		cc.			mg. per 100 cc.
Apr. 19	I	None.		9.00 a.m. 9.30 " 10.00 " 10.30 " 11.00 " 11.30 "	95 91 95 100 105 105
Apr. 21	V*	10	9.50 a.m.	9.15 a.m. 9.30 " 10.20 " 11.00 " 12.00 noon.	133 140 125 125 125
Apr. 22	VI	10	9.40 a.m.	8.10 a.m. 9.10 " 10.10 " 10.40 " 11.10 "	91 93 133 133 133
Apr. 23	VII	10	10.30 a.m.	9.30 a.m. 9.45 " 11.00 " 12.00 noon. 2.00 p.m.	72 87 133 133 111
Apr. 28	III	10	9.15 a.m.	8.30 a.m. 9.30 " 10.00 " 11.00 " 12.00 noon.	95 114 105 100 103
May 3	V	10	9.40 a.m.	9.10 a.m. 10.00 " 11.00 " 2.00 p.m.	95 108 118 111

\* After a fast of 48 hours; in all other cases the animals were fasted for 24 hours preceding the experiment.

TABLE III.

*Effect of Subcutaneous Injections of Urease Solution\* on the Blood Sugar of Fasted Rabbits.*

Date.	Rabbit No.	Urease solution injected.	Time of injection.	Time when blood was taken.	Glucose.
		cc.			mg. per 100 cc.
May 4	VII	1	9.20 a.m.	9.05 a.m.	77
				9.40 "	77
				10.20 "	77
				11.00 "	80
				2.00 p.m.	100
May 5	VI	2	9.20 a.m.	9.15 a.m.	80
				10.20 "	200
				2.00 p.m.	100
				4.00 "	80
May 6	IV	4	9.15 a.m.	9.10 a.m.	95
				10.30 "	100
				12.00 noon.	100
				2.00 p.m.	111

\* Arlco urease diluted 1:100.

TABLE IV.

*Effect of the Addition of Glucose to Rabbit Blood Filtrates\* on the Urea Nitrogen.†*

Rabbit No.	Blood filtrate.	Glucose content of normal blood.	Glucose added.	Urea nitrogen.
	cc.	mg. per 100 cc.	mg.	mg. per 100 cc. of blood
VIII	5	107	None.	9.6
	5	107	0.2	10.4
IX	5	115	None.	6.2
	5	115	10.0	6.74

\* Prepared according to Folin and Wu.

† Determined by the aeration method.

ammonium carbonate were followed by a rise in the blood sugar in case the initial value was low, and by a drop, if it was high. Subcutaneous injections of urease solution showed in two cases a small rise and in one case a marked increase in the blood sugar (Table III). The addition of glucose to blood filtrates resulted in an increase of the urea nitrogen figures (Table IV).

#### DISCUSSION.

According to Bang (2), the average blood sugar content of rabbits may be said to be 100 mg. per 100 cc. with a fluctuation from 80 to 130 mg., the latter occurring rarely. "A blood sugar of 140 mg. is to be considered as hyperglycemia." Our figures for normal rabbits after a 24 hour fast are in general close to the lower limit, with the exception of Rabbit V (Table II) in which the initial glucose content was 133 to 140 mg.

Bang showed that in rabbits immediately after bleeding the blood sugar goes up very rapidly and in 15 to 30 minutes reaches a maximum. It remains elevated or falls only slightly for about 1 hour, going down rapidly in the next 2 or 3½ hours. At the maximum the blood sugar reaches, according to Erlandsen (5), a level of 330 mg. per 100 cc. In Andersson's (1) experiments the blood sugar rose from 140 to 230 mg. in 5 minutes. Andersson's conclusion is that "the rise of the blood sugar seems to be independent of the amount of blood taken at the first bleeding."

If this statement held, it would render meaningless all the experimental work done on blood sugar, in which blood was drawn repeatedly. Fortunately Bang's, Andersson's, and Erlandsen's high blood sugar figures seem to be due to defects in their technique, since the carotid artery of the rabbits was always separated and a cannula introduced therein in order to obtain the desired amount of blood. Such manipulation is to be expected to raise the blood sugar not only in such a sensitive animal as the rabbit but also in any experimental subject. Moreover, Erlandsen and others took at the first bleeding an average of 25 gm. of blood, which is extremely high. The minimum amount withdrawn was 9.1 gm. This explanation is supported by Andersson's finding that bleeding from the ear vein gives a much smaller rise in the blood sugar, only to 160 to 170 mg. per 100 cc.

In our experiments, in which all these complicating factors were

avoided (even the fixing on the board was omitted), the blood sugar was satisfactorily constant even after repeated bleeding.

After the 10 cc. of ammonium carbonate solution were injected, no symptoms of any effect were observed. Convulsions and excitement are therefore excluded as factors in the results. The data of Table I, in which the dose is much higher, give further support to this observation. But the possibility remains that ammonium carbonate, being decomposed in the subcutaneous tissue with the liberation of ammonia, may have caused local pain, thus influencing the glucose content. This objection is avoided in the experiments with urease injections (see Table III), in which this event could not take place.

The experiments with ammonium carbonate, and with urease injections by which ammonium carbonate is liberated in the blood and tissues, are not essentially different, because the state of ammonia and ammonium salts in the fluids of the body depends upon the reaction of those fluids. At the reaction of the arterial blood most of the ammonia is present as  $\text{NH}_4$  ion, while a small part (about 0.5 per cent) exists as free base. The more alkaline the reaction, the greater the amount of free base present and *vice versa* (Trendelenburg (13)). Therefore, the rise in the glucose of the blood after ammonia or urease injection can only be attributed to the direct action of ammonia.

In the present state of our knowledge it is not possible to state the mechanism by which ammonia produces hyperglycemia. Witzemann and Livshis (14) found *in vitro* that insulin is more or less completely inactivated at room temperature by 0.5 or 0.7 N ammonium hydroxide in the course of some days and that its original activity is usually quickly restored on acidifying the ammoniacal solution with hydrochloric acid. A similar but more prompt action of ammonia might be expected to take place in a living organism, as it apparently did in our experiments. But Witzemann and Livshis' experiments are not conclusive because they injected the so called "inactivated" insulin with the ammonia and, therefore, the resulting blood sugar represents the summarized effect of the ammonia hyperglycemia (shown by us) and of the insulin hypoglycemia. The acidified ammonia-insulin mixture gives rise to a different effect, because the physiologically alkaline ammonium hydroxide and the physiologically

acid ammonium chloride formed have different effects on the organism. According to Fröhlich and Pollack (cited by Trendelenburg) in the isolated perfused frog liver the glycolytic effect of adrenalin solution is inhibited by ammonium chloride. If this is so, it is evident that a mixture of insulin and ammonium chloride, injected together, may cause a much more marked hypoglycemia than insulin alone, since the ammonium chloride may inhibit the antagonistic action of adrenalin.

Moreover, in the experiments of Witzemann and Livshis, very high blood sugar contents (up to 200 mg. per 100 cc.) are taken as "normal" in rabbits. Evidently the first bleeding had brought about an increase in the sugar of the blood taken 2 hours later, thus interfering with the effect of insulin. In Experiment 4 (May 16) the blood sugar even showed a rise after the injection of the insulin-ammonia mixture, a phenomenon which can in no way be attributed to insulin.

The possible ways in which ammonia may affect the carbohydrate metabolism are discussed in a previous paper (8).

It is evident from our experimental data that ammonia must be taken into consideration as a factor capable of influencing the glucose level of the blood. The hyperglycemia may be interpreted as a protective reaction of the organism against the excess of ammonia present which results in the formation of urea as shown by Fosse (6).

The liver is the chief site of ammonia detoxication, of the liberation of glucose, and of urea formation. Marie (11) described the important rôle of carbohydrates in the synthesis of urea. The addition of adrenalin to a rabbit liver is followed by a marked rise in urea, due partly to its glycolytic action. In case the liver has lost this capacity a severe intoxication develops. Matthews (12) in his investigation on Eck fistula dogs, fed on meat, found about ten times the normal amount of ammonia in the blood. Symptoms of ammonia intoxication occurred, and it is of great interest that these were relieved by glucose.

According to Mann and Magath (10) the total removal of the liver is followed by a definite and progressive decrease in blood sugar. Coordinate with this decreasing blood sugar, a characteristic group of symptoms develops. If glucose in suitable amounts is injected intravenously into an animal exhibiting these characteristic symptoms, it is immediately restored to normal.



Ringer ingeniously added glucose to his solution not only as a nutritive but also as a detoxicative substance. The good effect of glucose administration in therapeutics may be partly explained by this detoxicative property.

Fosse (7) demonstrated that *in vitro* the formation of urea by oxidation of proteins is very small, but if these are oxidized simultaneously with glucosc, considerable amounts of urea are formed. This fact, together with the data represented in Table IV, shows that the determination of urea nitrogen by the aeration method may give falsely high results due to the formation of additional urea during the process. The distillation method is, therefore, the only strictly accurate one.

#### SUMMARY AND CONCLUSIONS.

1. Moderate bleeding, performed with care, does not affect essentially the blood sugar level in rabbits.

2. Subcutaneous injections of ammonium carbonate or urease solution tend to produce hyperglycemia.

3. Hyperglycemia may be considered as a means of protection against increases in ammonia in the blood. This forms a basis for the use of glucose in cases of ammonia intoxication.

4. The blood sugar level may be partly regulated by ammonia.

5. In the aeration method some "extra urea" is formed in blood filtrates, thus giving falsely high figures.

6. The effect of ammonium hydroxide on insulin is still open to question.

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## THE RELATION BETWEEN PEROXIDATION AND ANTIRACHITIC VITAMIN.\*

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### INTRODUCTION AND HISTORY.

In the light of our knowledge concerning the nature of the antirachitic vitamin and its relation to ultra-violet rays on the one hand and the relation of sunlight and ultra-violet rays to the maintenance of calcium equilibrium in animal nutrition on the other hand, a relation between antirachitic vitamin and calcium equilibrium must be recognized. The evidence of the relation comes to us in recent investigations in calcium equilibrium by Hart and Steenbock and coworkers (1) with dairy cows and milk goats, Hughes and Payne (2) with laying hens, and Husband, Godden, and Richards (3) with pigs. The problem of calcium equilibrium in which this station has been interested is therefore a problem fundamentally involving antirachitic vitamin.

It has been known for a long time that sunlight causes an evolution of appreciable amounts of peroxide from exposed surfaces of a large variety of organic products. More recent work shows that this property is pronounced in fats and oils and especially marked on exposure to ultra-violet light which is now known to induce antirachitic potency in certain inactive substances and to cause evolution of peroxide vapor from the same and similar substances.

In an extensive investigation concerning the photochemically active emanation from various organic products, Russell (4) found a direct relation existing between sunlight and the emanation and produced evidence that hydrogen peroxide was the substance

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evolved. Kugelmass and McQuarrie (5) in studying the relation between radiant energy and antirachitic remedies such as cod liver oil, working with the alkaline oxidation of these products, found an evolution of reducing vapors and concluded that the phenomenon was probably of the nature of the "Russell effect." Clover (6) studied the oxidation and decomposition products of various ethers exposed to sunlight and isolated certain organic peroxides from them. Stutz, Nelson, and Schmutz (7) recently reported an investigation concerning the evolution of hydrogen peroxide from drying oils on exposure to light of various wavelengths and found that the blue and ultra-violet rays are most effective in this respect. It was of interest therefore to investigate peroxidation in the light of antirachitic potency.

We may include in a category of things known about the antirachitic factor; (a) stability under treatment with hydrogen sulfide, formaldehyde, sulfur dioxide, and hydrogen peroxide and instability under agitation with dilute acid, under treatment with steam, and with nitrous oxide as reported by Bills (8); (b) stable in non-aqueous alkali; (c) soluble and amenable to concentration in unsaponifiable fat; (d) formed by the action of ultra-violet rays in certain fresh oils and other products including peanut oil, oleic acid (9), olive oil, cottonseed oil, linseed oil, corn oil, butter, dried milk, spinach, and the crystalline sterols (10-14); (e) not formed on irradiation in old acid oils, mineral oil, dihydrosterols (10,15) water, casein, hardened peanut oil nor in stearic acid (9); (f) lost from irradiated sterols by recrystallization and aging; (g) not transferred from irradiated sterols to the aqueous filtrate (15); (h) formed in acylated and benzylated sterols on irradiation (10, 15); (i) can be administered effectively subcutaneously as irradiated cholesterol (12).

What may be termed an associated property of the antirachitic vitamin is the color reaction perfected and used by Poulsson and Weidemann (16) with which a transient blue-violet color is obtained with chloroform solutions of vitamin A-potent substances upon the addition of sulfuric acid. The intensity of the color was found to correlate closely with the vitamin A potency of cod liver oil preparations.

In the work reported in this paper an endeavor was made to examine for peroxidation those products known to have been

animal-tested for antirachitic potency in so far as they were at hand. Other products were examined and included to show something concerning the distribution of peroxidation in matter.

We prefer to use the term "peroxidation" rather than "autoxidation" because the former implies a content of unstable and reactive oxygen which can form hydrogen peroxide in the presence of water and liberate iodine from hydriodic acid.

The vitamin potency considered in this paper is the ability of the substance to prevent and cure rachitic lesions and to produce growth in animals on a rachitic diet.

#### EXPERIMENTAL.

In a search for a simple and rapid process for the examination of a product for peroxidation, it was found that the well known starch and hydriodic acid reaction with peroxides is generally applicable and is sufficiently delicate in aqueous emulsion. The relative amount of peroxide can also be roughly estimated.

The procedure followed in making the test for peroxide was such as to make comparison with a blank or check possible. Test-tubes, uniform as to size and color of glass, were used altogether. To a mixture of 0.5 cc. of a fresh 2 per cent solution of soluble starch, neutral or faintly acid in reaction, and 1 cc. of 2.5 per cent hydriodic acid, was added 1 gm. of the substance to be tested. The two layers were jarred into a mixture or a coarse emulsion and agitated for a minute. The presence of peroxide becomes evident by the appearance of a faint blue after 1 or 2 minutes in highly peroxidized oils and in 10 minutes in faintly peroxidized oils. At the end of 10 minutes the result was recorded.

#### *I. Peroxidation in Miscellaneous Products.*

Kugelmass and McQuarrie (5) produced evidence that cod liver oil evolves peroxide vapors which reduce a photographic plate. It would therefore be expected to liberate iodine from hydriodic acid producing a blue coloration with starch. 1 gm. of the oil produced such a depth of color that cod liver oil was used as a standard of comparison for other samples.

Among the substances tested as listed in Table I there is a noticeable correlation between potency and peroxidation in those

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substances which the literature shows to have been animal-tested. There is some question concerning the antirachitic potency of olive oil which showed slight peroxidation. Mineral oil of the brand Stanolax was found to be antirachitically inactive by Steen-

TABLE I.  
*Peroxidation in Miscellaneous Products.*

	Peroxidation.	Potency.	Literature.
Cod liver oil, old.....	++++	++++	(10, 17-19)
“ “ “ fresh.....	++++	++++	(10, 17, 18)
Linseed oil.....	—	—	(13, 15)
Sesame “ .....	—	—	(19)
Olive “ .....	+	-(?)	(10, 13, 19)
Mineral oil, liquid petro- laturum Merck.....	+	—	(10, 13)
Mineral oil, Stanolind....	—	—	(10, 13)
Corn oil.....	—	—	(10, 19)
Cottonseed oil.....	—	—	(10, 13)
Oleic acid.....	—	—	(15)
Butter, creamery, No. 1...	—	—	(18-20)
“ “ “ 2...	—	—	(18-20)
Milk.....	—	—	
Glycerol.....	—	—	(12, 21)
Wheat embryo oil, old....	—	—	
“ “ “ new....	—	—	
Cholesterol, 13 years old, old, m.p. about 135°....	++++	-(?)	(15, 22)
Cholesterol, recrystallized once, m.p. 143°.....	+	—	(15)
Cholesterol, recrystallized three times, m.p. 148°...	—	—	(15)
Benzene.....	—		
Cholesterol, fresh, m.p. 145°, 0.5 gm. in benzene	—	—	(10, 12-14)
Turpentine.....	++++		
“ freshly dis- tilled.....	++++		

bock and Black. A brand of mineral oil, Stanolind, showed no peroxidation while another brand, a much older sample, liquid petrolaturum Merck, showed some peroxidation.

In consideration of the work of Hess and Weinstock (13, 15) and others on cholesterol the results of the examination of an old

sample of cholesterol are particularly interesting. The sample tested was some of Kahlbaum's cholesterin and was at least 13 years old and had been stored in a cork-stoppered bottle in a glass case. It was light yellow in color, had some odor, and had a low melting point. This should have been inactive in accordance with work on aging of activated cholesterol carried out by Hess and coworkers, yet it showed as much peroxidation as cod liver oil. On four recrystallizations its reducing power was lost and its melting point raised to that of pure cholesterol. Hess found that the activity of cholesterol is lost through several recrystallizations. Another sample furnished by Professor V. E. Nelson, freshly prepared from sheep brains and recrystallized from acetone, when examined in benzene solution showed no peroxidation.

It should be noted that turpentine is peroxidized like cod liver oil. One distillation does not noticeably reduce the content of peroxidized constituents.

## *II. Effect of Ultra-Violet Light on Peroxidation.*

Since Baughman and Jamieson (23) and Stutz, Nelson, and Schmutz (7) working with a number of oils and Kugelmass and McQuarrie working with cod liver oil found a decided increase in the evolution of hydrogen peroxide vapors in these oils on exposure to sunlight and ultra-violet light, it was of interest to examine a number of similar products which have been shown by physiological chemists to be made antirachitic when similarly exposed to such radiant energy.

Exposure of a number of fats and oils was made at a distance of 30 cm. under a horizontal quartz mercury vapor lamp operating under a 110 volt, 60 cycle circuit, in the experiments listed in Table II. Examination for peroxidation was made immediately after irradiation.

The results indicate correlation of peroxidation with irradiation in all samples which have been found to acquire potency by irradiation. Benzene and glycerol do not peroxidize as a result of irradiation. The apparent exceptions to complete correlation between peroxidation and antirachitic potency as recorded in the literature are the two brands of mineral oil. These show different degrees of peroxidation, the fresher oil showing only an appreciable peroxide content whereas the older oil gave a stronger, positive reaction.

TABLE II.  
*Effect of Irradiation on Peroxidation.*

	Time of irradiation.	Peroxidation.	Potency.	Literature.
	<i>hrs.</i>			
Olive oil.....	0.5	++	+++	(10)
Cottonseed oil.....	0.5	+	+++	(13)
Corn oil.....	0.5	++	+++	(10)
Linseed oil.....	0.5	++	+++	(12, 13)
Glycerol.....	0.5	—	—	(12, 21)
Mineral oil, liquid petrolatum Merck.....	1.0	++	—	(10, 13)
Mineral oil, Stanolind.....	0.5	+	--	(10, 13)
Wheat embryo oil.....	1.0	++++	(?)	
Oleic acid, rancid.....	1.0	++++	—+(?)	(9, 10)
“ “ redistilled.....	0.5	++	—+(?)	(9, 10)
Sesame oil.....	1.0	++++	(?)	
Cholesterol, m.p. 148°C., recrystallized, 0.2 gm.....	0.5	—	+++	(10, 12-15)
Cholesterol, m.p. 148°C., recrystallized, 0.2 gm.....	1.5	—	+++	(10, 12-15)
Cholesterol, m.p. 148°C., recrystallized, 0.2 gm.....	2.5	+	++++	(10, 12-15)
Cholesterol, m.p. 148°C., recrystallized, 0.2 gm.....	10.0	++	++++	(10, 12-15)
Benzene.....	1.0	—	—	
Cholesterol, fresh, m.p. 145°C., recrystallized, 0.5 gm. tested in benzene.....	1.0	+++	+++	(10, 12-15)
Cholesterol, fresh, m.p. 145°C., recrystallized, 0.5 gm., in benzene.....	1.0	+++	+++	(10, 12-15)
Cholesterol, fresh, m.p. 145°C., recrystallized, 0.5 gm., in benzene in nitrogen.....	1.0	+++	+++	(10, 12-15)
Cottonseed oil.....	10.0	++++	—	(10, 13, 15)
Mineral oil, liquid petrolatum Merck.....	10.0	++++	—	(10, 13, 15)
Cod liver oil.....	10.0	++++	—	(10, 13, 15)
Olive oil.....	10.0	++++	—	(10, 13, 15)
Linseed oil.....	10.0	++++	—	(10, 13, 15)
Cottonseed oil in filled and stoppered Pyrex flask.....	2.0	+	++	(13)
Mineral oil, Stanolind, in filled and stoppered quartz flask...	1.5	+	—	(10, 13)

Oleic acid, whether rancid or purified, when examined after irradiation with ultra-violet light showed considerable peroxidation. There is disagreement in the literature concerning the antirachitic potency of irradiated oleic acid. Von Euler, Widell, and Erikson (9) report resumption of growth in rats stunted on a vitamin-free ration when irradiated oleic acid was added. The observation of Lifschütz (24) that cholesterol is formed in minute amounts when oleic acid is oxidized with permanganate is interesting in this connection.

Cholesterol when examined in crystalline form in portions of 0.2 gm. shows no peroxidation until it has been irradiated more than 2 hours, hence no peroxidation was reported in a sample of cholesterol which should have antirachitic potency. We must attribute a negative result here to a limitation of the method to solutions or liquids and also to a smaller response from an old recrystallized cholesterol than from a fresh sample. A fresh sample irradiated for one-half of the time chosen by Hess and Weinstock to be the optimum for activation, tested in benzene solution, indicated more peroxidation than the same amount of cod liver oil.

Hess and Weinstock (12) irradiated cholesterol and a vegetable oil in nitrogen and found them to be activated. The conclusion therefore was that oxygen plays no part in the process of activation. There was no noticeable difference in the response to the test for peroxidation whether cholesterol in a quartz flask was irradiated in air or in nitrogen purified according to the precautions observed by these authors. Cottonseed oil and fresh mineral oil also gave similar positive tests for peroxidation when irradiated in the absence of air as well as in the presence of air. These experiments cannot be interpreted to indicate presence of dissolved or combined oxygen during irradiation in view of the work of Hess and Weinstock on cholesterol. The results indicate that peroxidation is a combined result of irradiation and subsequent exposure to air through agitation.

Steenbock and coworkers (25) have found that activated vegetable oils are inactivated when excessively irradiated. Hess reports that activated cholesterol becomes inactive after an extended exposure to intense ultra-violet light. It was of interest therefore to examine for peroxidation some of these oils after



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exposure to ultra-violet light for 10 hours. Peroxidation was found to be much greater than when the oils were exposed for  $\frac{1}{2}$  hour. In the case of cholesterol an appreciable increase was noted. The oils irradiated for this length of time should have little or no antirachitic potency yet they show a large amount of peroxidation, indicating that the formation of hydrogen peroxide and reactive oxygen as the end-products of peroxidation, is the process by which the activity is destroyed.

TABLE III.  
*Effect of Heating and Aging on Peroxidation.*

	Peroxidation.	Potency.	Literature.
Cod liver oil, heated 6 hrs.....	+++++		
Mineral oil, liquid petrolatum Merck, heated 6 hrs.....	+++++		
Mineral oil, Stanolind, heated 6 hrs.....	+++++		
Olive oil, heated 5 hrs.....	+++++		
Cottonseed oil, heated 5 hrs.....	+++++		
Mineral oil, Stanolind, irradiated 1.5 hrs. in quartz flask.....	+	—	
Mineral oil, Stanolind, irradiated 1.5 hrs. in quartz flask, after 1 day.....	—	—	
Oleic acid, irradiated 0.5 hr. in air. ....	+	—+(?)	(9, 10)
“ “ “ 0.5 “ “ “ after 1 day.....	+	—+(?)	(9, 10)
Cottonseed oil, irradiated 2 hrs. ....	++	++	
“ “ “ 2 “ after 14 days.....	++	++	
Olive oil, irradiated 0.5 hr. ....	++	++	
“ “ “ 0.5 “ after 14 days	++	++	

We note that Mellanby (26) passed oxygen through cod liver oil for 4 hours at 120°C. without destroying the antirachitic vitamin and that McCollum and coworkers destroyed vitamin A in cod liver oil without destroying the antirachitic vitamin by passing air into the oil at 100°C. for 12 or 20 hours. On the other hand Bills (8) found the antirachitic factor to be destroyed by passing a current of steam through cod liver oil for 10 hours. It would appear then that water is a factor tending to destroy antirachitic vitamin under these conditions. It is known that organic peroxides are decomposed by water. Hence it is probable that in

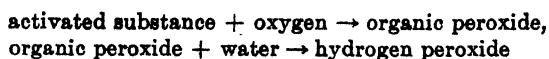
excessive irradiation where the temperature of the irradiated substance is not high enough to insure moisture-free conditions there is a change of organic peroxide to hydrogen peroxide and we get a positive reaction to the peroxidation test in the absence of vitamin D potency.

### *III. Effect of Heating and Aging on Peroxidation of Oils.*

Two vegetable oils, cod liver oil, and two brands of mineral oil were heated in small Jena glass flasks, loosely stoppered, on a steam hot plate at 105°C. for 5 to 6 hours to determine whether higher temperatures in the absence of ultra-violet light have any effect upon peroxidation in these oils. It has been noted by other investigators that irradiation is accompanied by a rising temperature. The oils all gave, relatively, a large positive response to the peroxidation test. This was an unexpected result in view of the absence of active rays of light. Since the temperature of the substances during irradiation in these experiments was about 40°C. the effects on peroxidation may be partially attributed to temperature effects on irradiated samples.

Since aging and deterioration of substances activated by irradiation have been found to occur it was desirable to determine to what extent the ability of some of these oils to peroxidize persists. A remarkable difference exists in this respect between fresh mineral oil and other oils known to be activated antirachitically by ultra-violet light. Its ability to respond positively to the peroxidation test does not persist for more than a day after irradiation while olive oil and cottonseed oil gave about the same peroxidation after 14 days. This fact explains the apparent discrepancy in correlation between capacity to peroxidize and potency in irradiated substance noted before.

These results do not support a theory that peroxide content and antirachitic vitamin are identical or even correlated because extensive peroxidation has been demonstrated in substances, which in the light of previous investigation with animals should be devoid of antirachitic potency. If an unstable molecular condition prerequisite to potency and induced by ultra-violet light can be conceived such that oxygen may combine to form an organic peroxide under certain conditions of exposure, thus,



we have an explanation for the phenomena so far described in connection with antirachitic vitamin. The results support a theory, however, that the inherent capacity, a potential property of a substance to peroxidize, is a measure of its antirachitic potency. Further, we have support for the following conclusion: The capacity to peroxidize is a function of the antirachitic potency.

## SUMMARY.

An examination of a number of vegetable oils, cod liver oil, and cholesterol for peroxidation shows (1) correlation between apparent antirachitic potency and peroxidation in untreated samples, (2) correlation between apparent potency and peroxidation in irradiated samples, and (3) no correlation between apparent potency and peroxidation in excessively irradiated samples.

Heating oils in air causes a relatively large amount of peroxidation.

Fresh mineral oil does not maintain its capacity to peroxidize, whereas irradiated vegetable oils maintain that capacity.

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## THE EFFECT OF MOLECULAR COMPLEXITY ON THE END-PRODUCTS FORMED BY CLOSTRIDIUM THERMOCELLUM.\*

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In a previous paper (1) we have described the fermentation products formed from cellulose by *Clostridium thermocellum*. It was shown that in 4 to 7 days this organism brings about a rapid and nearly complete destruction of a 2 per cent cellulose medium. The principal products formed were identified as acetic acid, ethyl alcohol, carbon dioxide, and hydrogen. A trace of butyric acid was also found but no lactic acid could be obtained, although large volumes of culture were examined for this acid. It seemed probable that the same products would be formed from the fermentation of the common sugars, and other organic compounds.

The preliminary tests indicated that all of the sugars were not fermented in the same way. On galactose, particularly, the fermentation appeared irregular. An unusually rapid and heavy growth was obtained in the absence of  $\text{CaCO}_3$  and almost complete disappearance of the sugar. Without  $\text{CaCO}_3$  in the media the organism showed little ability to ferment the other monosaccharides. The growth on lactose was very much like that on galactose. These differences in behavior seemed to warrant a more extended study of the fermentation of the sugars and related compounds. The result has been entirely unexpected and indicates that the structure of the compound exerts a profound effect on the way in which the microorganism breaks up the molecule and on the resulting end-products. This thermophilic micro-

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organism exhibits a remarkable range of fermentation powers and versatility in its manner of attacking the various carbohydrates.

#### EXPERIMENTAL.

*Medium and Fermentation.*—The nutrient salts and peptone were the same as described in the previous paper (1). The sugars were sterilized separately from the salts and peptone and were then added to the latter with a sterile pipette to make a concentration of about 2 per cent. This medium was inoculated with 1 per cent of a 24 hour culture of the bacteria. Sterilized calcium carbonate was then added to maintain a suitable hydrogen ion concentration and the flasks tightly capped with tin-foil and incubated at 65°C. for 5 to 7 days. Evolution of gas usually began in a few hours and in some cases a foam of an inch or more covered the surface of the fermenting liquid for several days. The flasks were shaken daily during the incubation period to bring about a better neutralization of acids by the calcium carbonate. When gassing ceased, usually after 5 to 6 days, the cultures were analyzed for acids and alcohol.

In one experiment the fermentations were carried out in flasks equipped with an absorption trap for collecting the carbon dioxide (2). In this case sterilized bromocresol purple was added to the fermenting liquid and the acidity neutralized daily with sterilized  $N$  NaOH.

*Methods of Analysis.*—The cultures were analyzed for unfermented sugar, volatile acid, non-volatile acid, alcohol, and carbon dioxide by methods which have already been described in previous publications, (2-5).

*Fermentation Products.*—The results of the analyses are given in Table I and have been arranged in the following order: monosaccharides, polysaccharides, and other fermentable compounds such as mannitol, etc. The unfermented residue, if any, has been deducted from the initial concentration to give the weight of compound fermented in Column 2. The weight of fermentation products is totaled in Column 6, and the percentage of sugar, etc., accounted for by this total is given in the last column. A glance at this column shows that from 45 to 96 per cent of the sugar is accounted for by the three fermentation products. It is obvious that the sugars are broken down in more than one way.

The most outstanding and noteworthy product is lactic acid.

It should be borne in mind that from cellulose only uncertain evidence of the formation of lactic acid could be secured. In the fermentation of the monosaccharides it is the product formed in largest amount from four of the six sugars and comprises nearly 20

TABLE I.  
*Fermentation Products from Sugars and Other Organic Compounds.*  
*After 6 Days at 65°C.*

Compound.		Acids.		Alcohol as ethyl.	Total products.	
Kind.	Amount fer- mented.	Volatile as acetic.	Non- volatile as lactic.		Weight.	Percent- age of compound fer- mented.
(1)	(2)	(3)	(4)	(5)	(6)	(7)
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	
Arabinose . . . . .	1.68	0.239	0.844	0.109	1.192	71
Xylose . . . . .	2.13	0.392	0.767	0.263	1.422	67
" . . . . .	1.76	0.225	0.679	0.106	1.010	57
Rhamnose . . . . .	1.53	0.791	0.265	0.291	1.347	88
" . . . . .	1.46	0.517	0.316	0.116	0.949	65
Glucose . . . . .	2.29	0.216	0.609	0.209	1.034	45
" . . . . .	2.29	0.204		0.241		
" * . . . . .	0.80	0.118	0.324	0.101	0.543	68
Fructose . . . . .	1.76	0.222	1.340	0.126	1.688	96
" . . . . .	1.39	0.095	1.062	0.083	1.240	89
" † . . . . .	1.30	0.165	0.820	0.165	1.131	87
Galactose . . . . .	1.77	0.752	0.036	0.368	1.156	65
Lactose . . . . .	1.83	0.747	0.133	0.357	1.237	68
Whey . . . . .	2.22	0.707	0.259	0.560	1.526	69
Raffinose . . . . .	2.35	0.837	0.070	0.488	1.395	59
Starch . . . . .	2.42†	0.516	0.021	0.456	0.993	
Mannitol . . . . .	2.42†	0.454	0.253			
Salicin . . . . .	2.42†	0.199	0.180	0.066		
Malic acid . . . . .	2.42†	0.046		0.008		

\* 0.258 gm. of CO<sub>2</sub>

† 0.116 gm. of CO<sub>2</sub>.

‡ Weight before inoculation; weight of fermented material not determined.

per cent from a fifth sugar, rhamnose. Fructose is conspicuous by the fact that more than 75 per cent of the sugar is converted into lactic acid. Almost all of the fructose is accounted for in the three products given. There can be little or no formation of



carbon dioxide or hydrogen in the fermentation of this sugar. The absence of any noticeable evolution of gas during the fermentation furnished visible evidence of this fact. The paucity of gas formation and extensive lactic acid production is presumably associated with the molecular complexity. The presence of a ketone group first suggests itself but doubt arises that this is the cause when it is discovered that the aldehyde group is not the determining cause in the fermentation of the other sugars. The aldoses behave about the same with the exception of galactose. This sugar yields little or no lactic acid and unusually large amounts of acetic acid and ethyl alcohol. The kind and amount of fermentation products are practically the same as those from cellulose.

The polysaccharides derived from 2 or more sugar molecules such as lactose, raffinose, etc. behave more like galactose than like the other monosaccharides. As the compound becomes more complex, *e.g.* starch, the resemblance to the cellulose type of fermentation becomes greater. Clearly raffinose and starch are not hydrolyzed to the simple sugars before they are fermented. Glucose is apparently not an intermediate product in the fermentation of either starch or cellulose. Salicin, on the other hand, resembles the simple sugars in its yield of products. Low alcohol and good lactic acid production is shown here. Mannitol which is a simple compound, is like the simple sugars in the type of products formed from it. As the complexity of the molecule increases, it seems certain that the type of fermentation changes. On the other hand, malic acid which has the simplest structure of the compounds in Table I was not attacked by the bacteria. In this respect *Clostridium thermocellum* differs from the majority of bacteria. Malic acid is usually considered one of the most fermentable of the organic acids. A microorganism which can ferment such an unusual sugar as rhamnose and such a resistant compound as cellulose ought, it would seem, to be able to ferment malic acid. Citric acid was also tested but found unfermentable.

Glucose was prepared by the method of Benedict, Dakin, and West (6) and was found to be fermentable. The fermentation has not been thoroughly studied but the chief product appears to be volatile acid. The pentosans of corn stover, alfalfa, and commercial pectin were also attacked. From 31 to 78 per cent of the pentosans in these materials was destroyed.

*Identification of Volatile Acids.*—The volatile acids and the acids obtained by oxidation of the alcohols were subjected to a Duclaux distillation and the constants calculated. These figures are given in Table II and show that with the exception of mannitol the volatile acid is practically all acetic. The presence of a higher fatty acid is clearly indicated in the case of mannitol. On acidifying the barium salt an odor of butyric acid could be detected. The percentages of acetic and of butyric acid have been calculated from the Duclaux data by the method of Gillespie (7) and found to be 91.8 and 8.2 per cent respectively.

TABLE II.  
*Distillation Constants of the Volatile Acids by the Duclaux Method.*

Source of acid.	Fractions.									
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Volatile acid from:										
Xylose	7.6	15.8	24.1	32.9	41.9	51.2	61.8	72.9	85.2	100
Galactose	7.8	15.9	24.2	33.3	42.5	52.0	62.3	73.3	85.7	100
Fructose	7.7	15.9	24.2	33.0	42.1	51.4	61.5	70.1	85.3	100
Lactose	7.5	15.5	23.9	32.7	41.8	51.4	61.7	72.9	85.4	100
Mannitol	8.2	17.6	25.3	34.1	43.2	52.8	62.8	76.0	85.9	100
Alcohol from:										
Xylose	7.9	16.1	24.6	33.4	42.6	52.2	62.5	73.5	85.7	100
Rhamnose	10.5	21.1	31.6	41.8	51.8	61.7	71.4	81.1	90.5	100
Glucose	7.9	16.1	24.6	33.4	42.5	51.8	61.9	73.3	85.5	100
Fructose	7.9	16.1	24.1	33.5	42.7	52.4	62.5	73.6	85.7	100
Duclaux constant for acetic acid	7.4	15.2	23.4	32.0	40.9	51.5	60.9	71.9	84.4	100

The data for the acids from the alcohol agree with those for acetic acid with one exception and therefore prove that ethyl alcohol is the fermentation alcohol. The figures do not check exactly with Duclaux's figures. Our apparatus always gives slightly higher values for acetic acid than was reported by Duclaux. Rhamnose, however, is clearly out of line with the other sugars and evidently furnishes some higher alcohol. Qualitative tests on the oxidation acids showed butyric acid to be present. By calculation from the Duclaux data the percentage of ethyl alcohol was found to be 61.8 per cent and of butyl alcohol 38.2 per cent. Rhamnose is a methyl pentose. This peculiar structure evidently results in the forma-

tion of other products than those given by xylose and arabinose. The disposition of the methyl group is an interesting question which cannot be answered from the present data.

*Composition of the Barium Salts.*—As a check on the Duclaux data and in some cases where both determinations did not seem necessary the barium salts were analyzed. The salts were dried

TABLE III.

*Analysis of the Barium Salts of Fermentation Acids and Acids from Alcohol.*

Source of the salt.	Weight of salt taken.	Weight of BaSO <sub>4</sub> .	Ba content of salt.
	gm.	gm.	per cent
Volatile acid from:			
Rhamnose .....	0.5107	0.4427	51.0
Glucose .....	0.2025	0.1789	52.0
Fructose .....	0.9046	0.8144	52.9
Raffinose .....	0.5196	0.4733	53.8
Starch .....	0.3184	0.2776	53.2
Mannitol .....	0.2955	0.2578	49.4
Whey .....	0.5603	0.4112	53.9
Alcohol from:			
Rhamnose .....	0.2256	0.1897	49.5
“ .....	0.7300	0.5872	47.4
Galactose .....	0.2603	0.2375	53.7
Lactose .....	0.2401	0.2283	53.5
Raffinose .....	0.3386	0.3087	53.7
Starch .....	0.2957	0.2681	53.4
Whey .....	0.3060	0.2784	53.8
Non-volatile acid from:			
Xylose .....	0.1863	0.1373	43.3
Rhamnose .....	0.0988	0.0720	41.9
Fructose .....	0.1276	0.0939	43.1
Salicin .....	0.1133	0.0793	41.1
Theory for Ba in Ba(C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> ) <sub>2</sub> .....			53.8
“ “ “ “ Ba(C <sub>3</sub> H <sub>5</sub> O <sub>3</sub> ) <sub>2</sub> .....			43.5

to constant weight at 130°C. and converted into BaSO<sub>4</sub> by ignition in the presence of sulfuric acid, and the percentage of barium calculated from the weight of BaSO<sub>4</sub>. The data are given in Table III and tell the same story as the Duclaux figures. Acetic acid and ethyl alcohol comprise the volatile acid and the alcohol in the fermentation of most of the compounds. Mannitol and rhamnose are exceptions. Fermentation products of higher

molecular weight are demonstrated by the lower percentages of barium.

The barium content of the non-volatile acid from four of the fermented compounds is also given in Table III. The data agree well in two cases and depart slightly from the theoretical for barium lactate in two others. Too much reliance cannot be placed on these data owing to the fact that the crude barium salts were analyzed. These contained considerable pigment and other impurities. Better evidence as to the nature of the non-volatile acid was obtained from the zinc salts. The thiophene test for

TABLE IV.

*Forms of Lactic Acid as Determined by the Water of Crystallization of Their Zinc Salts.*

Source of salt.	Weight of hydrated salt.	Weight of anhydrous salt.	Water of crystallization.
	gm.	gm.	per cent
Arabinose.....	0.7785	0.6757	13.2
“.....	0.3101	0.2683	13.5
Xylose.....	0.7641	0.6650	12.3
“.....	0.4165	0.3594	13.9
Rhamnose.....	1.0003	0.8583	14.2
Glucose.....	0.6037	0.5263	12.8
Fructose.....	1.5672	1.3646	12.9
“.....	3.5192	3.0618	13.0
Mannitol.....	0.2666	0.2231	12.6
Theory for $H_2O$ in $Zn(C_3H_5O_2)_2 \cdot 2H_2O$ .....			12.9
“ “ “ “ $Zn(C_3H_5O_2)_2 \cdot 3H_2O$ .....			18.2

lactic acid was applied to all of the barium salts and gave positive results in all cases.

*Identification of the Non-Volatile Acid.*—The barium salts were converted into zinc salts by decomposing with  $ZnSO_4$ . The solution of zinc salt was bone-blackened and evaporated to crystallization at  $37^\circ C$ . When evaporation had brought the solution to a small volume (10 to 20 cc.) an equal volume of 95 per cent ethyl alcohol was added to insure more complete separation of the salt. In this way practically all of the zinc salt was obtained. After drying over calcium chloride to constant weight (12 to 24 hours) the water of crystallization was determined by drying to constant

weight at 105–107°C. The percentages given in the last column, Table IV, show that in most cases the zinc lactates contained about 13.0 per cent of water which is very close to that required for *d*- or *l*-zinc lactate, 12.9. To decide which of these two forms was present the specific rotation of the zinc lactates from three of the sugars was determined. All proved to be levorotatory and gave the following specific rotation,  $[\alpha]_D^{20}$ .

Zinc salt from fructose . . . . .	7.5
“ “ “ arabinose . . . . .	7.9
“ “ “ xylose . . . . .	7.7

The zinc salt from rhamnose also was levorotatory but its specific rotation was not determined. These rotations are in good agreement with those given by Hoppe-Seyler and Araki (8), Purdie (9), and Irvine (10) which range from  $\pm 7.52$  to  $\pm 7.85$ . Since the rotation of the zinc salt is opposite to that of the free acid it is evident that *Clostridium thermocellum* produces dextro-lactic acid.

#### SUMMARY.

The fermentative powers of the thermophilic microorganism *Clostridium thermocellum* have been tested on nine sugars and five related compounds. The outstanding fact in these tests is the fermentation of the methyl pentose, rhamnose. This sugar is destroyed apparently as rapidly as the more common sugars. Mannitol, starch, and salicin are also fermented, but malic and citric acids are not attacked to any extent.

The products formed from sugars, etc., are notably different from those produced from cellulose. Lactic acid, which is not formed from cellulose, is a conspicuous product from all the monosaccharides, with the exception of galactose. In the case of fructose it makes up more than 80 per cent of the total products and 75 per cent of the fermented sugar. The more complex compounds such as lactose and starch yield the same products as cellulose. The data point clearly to the complexity of the molecule as a factor in determining the type of fermentation.

The lactic acid consists almost entirely of the dextro form, the volatile acid is almost entirely acetic, and the alcohol is mainly ethyl. Rhamnose and mannitol appear to give some butyric acid and butyl alcohol.

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# GRAPHICAL INTERPRETATION OF ELECTROMETRIC TITRATION DATA BY USE OF COMPARISON CURVES.

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The titration constants<sup>1</sup> of a compound may be determined by simple formulas<sup>2</sup> or by the buffer value method of Van Slyke,<sup>3</sup> but for a compound such as nucleic acid which has many groups and which is difficult to obtain in a pure state, it seems expedient to use the graphical method herewith described, in which we compare the titration curve<sup>4</sup> of a substance with a curve calculated from known constants.

<sup>1</sup> See Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239. The titration constants are those obtained by calculating the titration data of an acid, base, or ampholyte, as if it were a mixture of monovalent acids. These constants ( $G'$ ) are related to the classical dissociation constants ( $K'$ ) by the following formulas.

$$K_1' = G_1' + G_2' + G_3' + \text{etc.} \quad (1)$$

$$K_1'K_2' = G_1'G_2' + G_2'G_3' + G_1'G_3' + \text{etc.} \quad (2)$$

$$K_1'K_2'K_3' = G_1'G_2'G_3' + G_1'G_2'G_4' + G_1'G_3'G_4' + \text{etc.} \quad (3)$$

etc.

$$K_2' = \text{equation (2) divided by (1)}$$

$$K_3' = \text{equation (3) divided by (2), etc.}$$

In general the titration constants are nearly identical with the dissociation constants. The prime marks indicate that the constants are not corrected for activity.

<sup>2</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, lxviii, 1239.

<sup>3</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525. Hastings, A. B., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 289.

<sup>4</sup> The titration curve consists of a curve in which the corrected equivalents of base ( $b'$ ) are plotted against  $\text{pH} = -\log H$ .

$$b' = \frac{b - a + h - oh}{c}$$

$b$  = concentration of strong base.

$a$  = " " " acid.

$h$  = " " hydrogen ions ( $H$  = the activity).

$oh$  = " " OH ions ( $OH$  = the activity).

$c$  = " " substance.



The curve for the unknown or impure substance we will call the *unknown curve* (*U*) and that having known constants, the *known curve*. The latter may be the curve of a substance whose constants are known and which is expected to be similar to the unknown substance; or the *known curve* may be calculated purely from constants predicted on the basis of chemical structure<sup>5</sup> but which have not been experimentally verified.

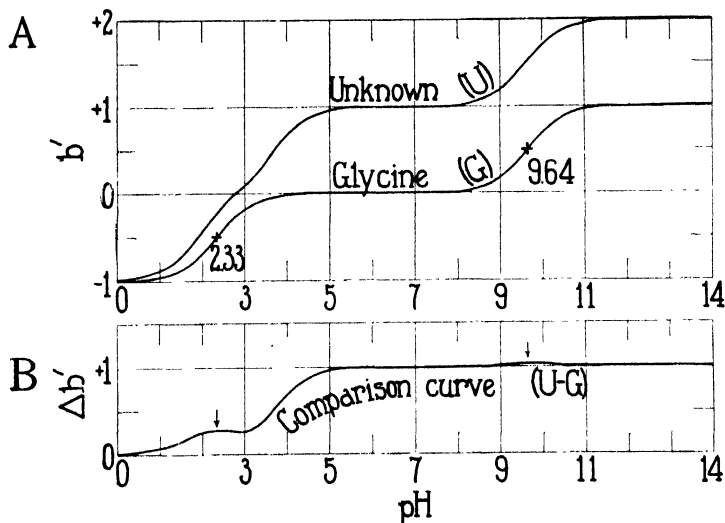


FIG. 1.

In the titration curves we plot the corrected equivalents of base ( $b'$ ) against pH.<sup>4</sup> Both the *known* and *unknown* curves are placed on the same piece of paper. If at various pH values we subtract  $b'$  of the *known* curve from  $b'$  of the *unknown* curve, we obtain a *comparison* curve which may be used to study the constants, the structure, and the state of purity of a substance.

As an aid in interpreting these *comparison* curves we give the following hypothetical examples.

#### Case I (Fig. 1).

In Fig. 1 are the titration curves of an "unknown" substance and also that of glycine. Subtracting the values of  $b'$  in the *known*

<sup>5</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, *xlvi*, 1251.

curve from those of the same pH values in the *unknown* curve we get the *comparison* curve ( $U-G$ ) in Fig. 1, B, which may be interpreted as follows:

1. At pH 0 this curve is at 0 equivalents; hence the *unknown* substance has the same number (one) of amino groups as glycine.

2. At pH 14 the curve is at 1.0; hence the unknown substance has one more acid group than glycine (making two acid groups).

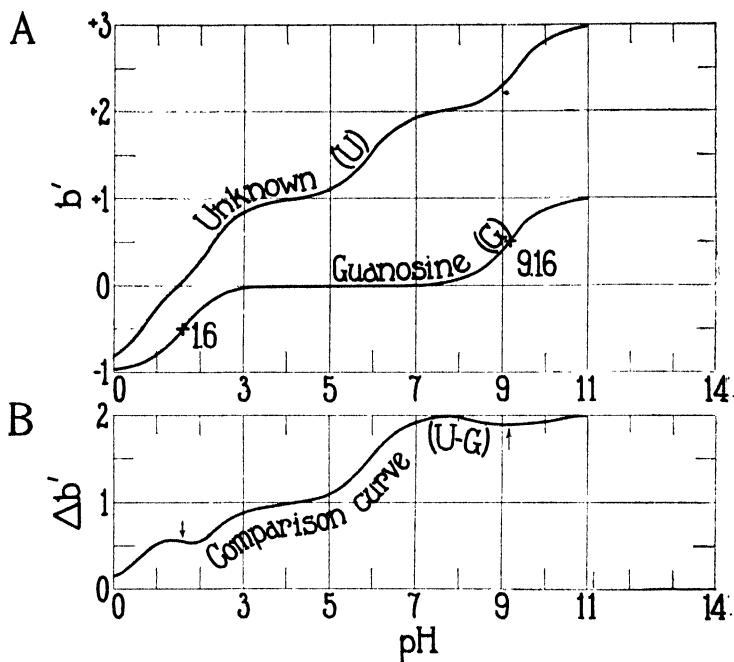


FIG. 2.

3. The rise of one equivalent occurs between pH 0 and 6. The upper part of this rise has the slope of a monovalent curve with  $pG' = 3.7$ . This is  $pG_{2u}'$  of the unknown substance.

4. At  $pG_1'$  of glycine (2.33) the value of  $\Delta b'$  on the comparison curve is about 0.25, and as an approximation we may assume that 2.33 is about 0.25 the distance in a horizontal direction between  $pG_{1u}'$  and  $pG_{2u}'$  of the unknown substance:

$$\Delta b' \text{ (at } pG_1') \quad \frac{pG_1' - pG_{1u}'}{pG_{2u}' - pG_{1u}'} \text{ (approximately)} \quad (4)$$

Hence  $pG_{1u}' = 1.9$ , since  $pG_{2u}' = 3.7$  and  $\Delta b' = 0.25$ .

5. The comparison curve is approximately horizontal in the range of the amino dissociation, indicating that this constant is about the same as that of glycine (9.64). However, the slight rise shows that it is slightly lower than 9.64. The height of the rise at pH 9.64 is equal to the vertical distance between the midpoint of one monovalent curve and another 0.04 pH unit below. Hence  $pG_{3u}' = 9.60$ .

We conclude that the unknown substance has two acid groups and one amino group with  $pG'$  values equal to: 1.9, 3.7, and 9.60. The first two constants correspond to two carboxyl groups in close proximity. The substance is aspartic acid.<sup>6</sup>

### *Case II (Fig. 2).*

The known curve is of guanosine, having an amino<sup>7</sup> group  $pG_1' = 1.6$  and a phenolic hydroxyl group  $pG_2' = 9.16$ . The curve is carried only to pH 11.

1. The comparison curve starts at 0 and ends at two equivalents. Hence there is one amino group (as in guanosine) and three acid groups (two more than in guanosine). This conclusion assumes that there is no free strong acid or base present.

2. One equivalent is reached at pH 4, and the latter part of this rise corresponds to a monovalent curve with  $pG' = 2.3$ .

3. At pH 1.6 ( $pG_1'$  of guanosine)  $\Delta b' = 0.56$ , hence from equation (4),  $pG_{1u}' = 0.7$ .

4. The rise between pH 4 and 8 is a monovalent curve with  $pG' = 5.92$ .

5. The drop in the curve at 9.16 ( $pG_2'$  of guanosine) corresponds to the vertical distance between the midpoint of a monovalent curve and a similar curve 0.20 pH unit higher. Hence  $pG_{4u}' = 9.36$ .

<sup>6</sup> In this case it would have been simpler and more accurate to have calculated the constants directly, but this illustrates the interpretation of a comparison curve. Sometimes, as in nucleic acid, the direct calculation is impracticable. Case III is one in which direct calculation would be impossible.

<sup>7</sup> The numerical value of  $pG_1'$  of guanosine is too low for a typical aromatic amino group. This indicates an abnormality in the mechanism of the ionization of the group such as to increase the work required for ionization.

We conclude that the substance has three acid groups and one amino group, with  $pG'$  values 0.7, 2.3, 5.92, and 9.36.

Knowing that phosphoric acid attached to a sugar group has dissociation constants at about 1 and 6 and that the presence of a charged group would raise the  $pG'$  of the amino group about 0.7 pH unit, we conclude that the chemical groups are respectively:

Phosphoric acid radical, first step,  $pG_1' = 0.7$ .

Aromatic amino group,  $pG_2' = 2.3$ .

Phosphoric acid radical, second step,  $pG_3' = 5.92$ .

Aromatic hydroxyl group,  $pG_4' = 9.36$ .

The substance is guanylic acid.

### Case III (Fig. 3).

The third and last case is somewhat different and demonstrates the utility of this method whereas the direct method of calculation of constants would lead to meaningless results. The known curve is cytidine phosphoric acid (*C. P. A.* in Fig. 3,A).

In order to make this a practical case the unknown curve is given only between pH 2 and pH 12.

The difference between the unknown and the known curves is shown in the first comparison curve,  $C_1$ , Fig. 3,B, which we may analyze as follows:

1. The most conspicuous characteristic of this curve is the drop about pH 6 which is the range of dissociation of the secondary phosphoric group of cytidine phosphoric acid. Concluding that this group does not exist in the unknown solution let us *add* a monovalent curve (*i.e.*, subtract the negative curve,  $-P''$ ) with  $pG' = 5.97$ . This gives us the second comparison curve,  $C_2$  in Fig. 3,C.

2. The second curve,  $C_2$ , very much resembles that of  $H_3PO_4$ , particularly since it has a  $pG' = 6.8$  which is that of the secondary ionization of  $H_3PO_4$ . Since the difference in  $\Delta b'$  from pH 4 to pH 9 is only 0.5 equivalent, we may *subtract* a curve for 0.5 equivalent of free  $H_3PO_4$  (*i.e.*, Curve  $P/2$ ). This gives the third comparison curve,  $C_3$  in Fig. 3,D.

3. The third comparison curve,  $C_3$ , is horizontal except at the ends. The value of  $\Delta b'$  ( $-1.25$ ) would indicate that the primary

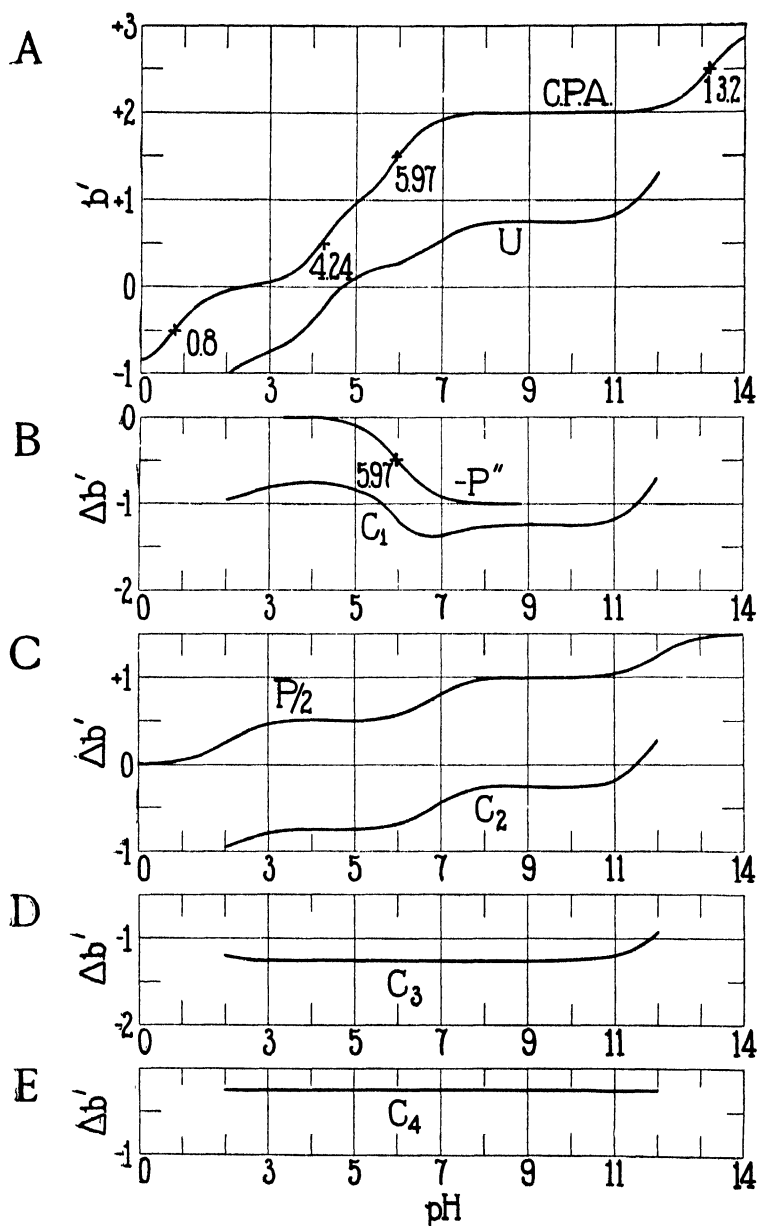


FIG. 3.

phosphoric group is missing. With this clue we would suppose the substance to be *cytidine* (instead of cytidine phosphoric acid). Hence let us *add* the primary group of cytidine phosphoric acid ( $pG' = 0.8$ ) and substitute the values 4.22 and 12.3 of cytidine for the values 4.24 and 13.2, respectively, of cytidine phosphoric acid (by *subtracting* the former and *adding* the latter). This gives the fourth comparison curve,  $C_4$  in Fig. 3, *E*.

4. The last comparison curve,  $C_4$ , is straight and horizontal. The value of  $\Delta b'$  ( $-0.25$ ) indicates the presence of a quarter equivalent of alkali (NaOH).

The final interpretation is that the unknown solution contains cytidine plus half an equivalent of free  $H_3PO_4$  and a quarter equivalent of NaOH.

#### CONCLUSIONS.

This method of interpretation of titration data may be applied in many other cases and is used in the following article on nucleic acid.

The elementary chemical analyses of unknown substances aid in the interpretation of titration curves and should agree with the conclusions.

In the above examples we have plotted the true values<sup>4</sup> of  $b'$  of the unknown substances in order to avoid the complication of determining the concentration ( $c$ ) of these substances. If we assume a given (but wrong) value of  $c$  we may calculate values of  $b'$  which must be multiplied by a factor in order to obtain the true curve. In Cases I and II this factor would be such as to make the change in  $b'$  from pH 7 to pH 12 equal to one equivalent. In Case III this factor would be hard to determine; but fortunately the factor happens to be about unity since the lower molecular weight is nearly compensated by the weight of impurities ( $H_3PO_4$  and NaOH).

#### SUMMARY.

The titration data of a complex or impure substance may be interpreted by a graphical method in which the titration curve is compared with a curve having known titration constants. Comparison curves are drawn and interpreted in the manner described. Three hypothetical examples are discussed in detail.



# NUCLEIC ACID STRUCTURE AS DETERMINED BY ELECTROMETRIC TITRATION DATA.

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## I. INTRODUCTION.

Nucleic acid is composed of four nucleotides joined together. In *animal nucleic acid* it has been shown, by chemical evidence, that the nucleotides are joined together by *ester* linkages between the phosphoric and carbohydrate groups.

The structure of *plant nucleic acid* is still in dispute. Jones believes that at least one of these linkages is an *ether* linkage, while Levene has proposed a structure in which *all* three linkages are *ester* linkages (as in animal nucleic acid).

With the hope of proving the structure by means of electrometric titration data we determined the dissociation constants of the four plant nucleotides (and also the nucleosides).<sup>1</sup> The corresponding animal nucleotides and nucleosides should have values almost identical with those from yeast.

We may estimate the titration constants<sup>2</sup> of nucleic acid (see Table I) in the light of formulas which have been derived for the relation between dissociation constants and structure,<sup>3</sup> and we may draw the theoretical titration curve. Figs. 1 to 3 give the theoretical curve for nucleic acid (plant or animal) according to Levene's structure. If Jones' structure for plant nucleic acid were correct, there would be another secondary phosphoric group having a  $pG'$  value equal to about 7.0.

In Figs. 1,A, 2,A, and 3,A are also the experimental electrometric titration curves of various samples of nucleic acid.

<sup>1</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxx, 519.

<sup>2</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

<sup>3</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1251.



By use of the comparison curve method described in the previous article<sup>4</sup> we may draw the comparison curves of these substances in Figs. 1,B, 2,B, and 3,B. The details of the interpretation of these curves are given later. The conclusions are as follows:

TABLE I.

*Predicted Titration Constants of Nucleic Acid Compared with the Corresponding Constants in the Nucleotides and Nucleosides.*

Group.			Symbol.	Nucleic acid.	Nucleo- tides.	Nucleo- sides.
Four sugar groups. ....			pG <sub>14</sub> '	>13	13.2-13.9	12.3-12.5
			pG <sub>13</sub> '	>13		
			pG <sub>12</sub> '	>13		
			pG <sub>11</sub> '	>13		
Range of titration data for nucleic acids.	Aromatic groups.	Uracil (or thymine).	pG <sub>10</sub> '	10.2	9.43	9.17
		Guanine...	pG <sub>9</sub> '	10.1	9.36	9.16
	Secondary phosphoric group.....		pG <sub>8</sub> '	6.0	5.9-6.0	
	Aromatic amino groups.	Cytosine...	pG <sub>7</sub> '	4.2	4.24	4.22
		Adenine...	pG <sub>6</sub> '	3.7	3.70	3.45
		Guanine..	pG <sub>5</sub> '	2.3	2.3	1.6
	Primary phosphoric groups (Step 4).....		pG <sub>4</sub> '	<2		
	Isoelectric point.....			pI	<2	1.5-4.5
Primary phosphoric groups.	Step 3.....	pG <sub>3</sub> '	<2	0.7-1.0		
	" 2.....	pG <sub>2</sub> '	<1			
	" 1.....	pG <sub>1</sub> '	<1			

If Jones' structure of yeast nucleic acid were correct, there should be another secondary phosphoric group, with a titration constant (pG') at about 7.0.

1. *Anhydride Formation.*—All the comparison curves are negative at pH 6. This indicates that some of the groups which "dissociate"<sup>5</sup> below this pH are not functioning. Probably some type of anhydride has formed.

2. *Yeast Nucleic Acid.*—Two samples of yeast nucleic acid show

<sup>4</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1926, lxx, 319.

<sup>5</sup> We use the word "dissociation" in the sense in which we have previously defined it;<sup>2,3</sup> namely, to apply to the *ionization* of an acid or acidic group, or the *hydrolysis* of a base or basic group.

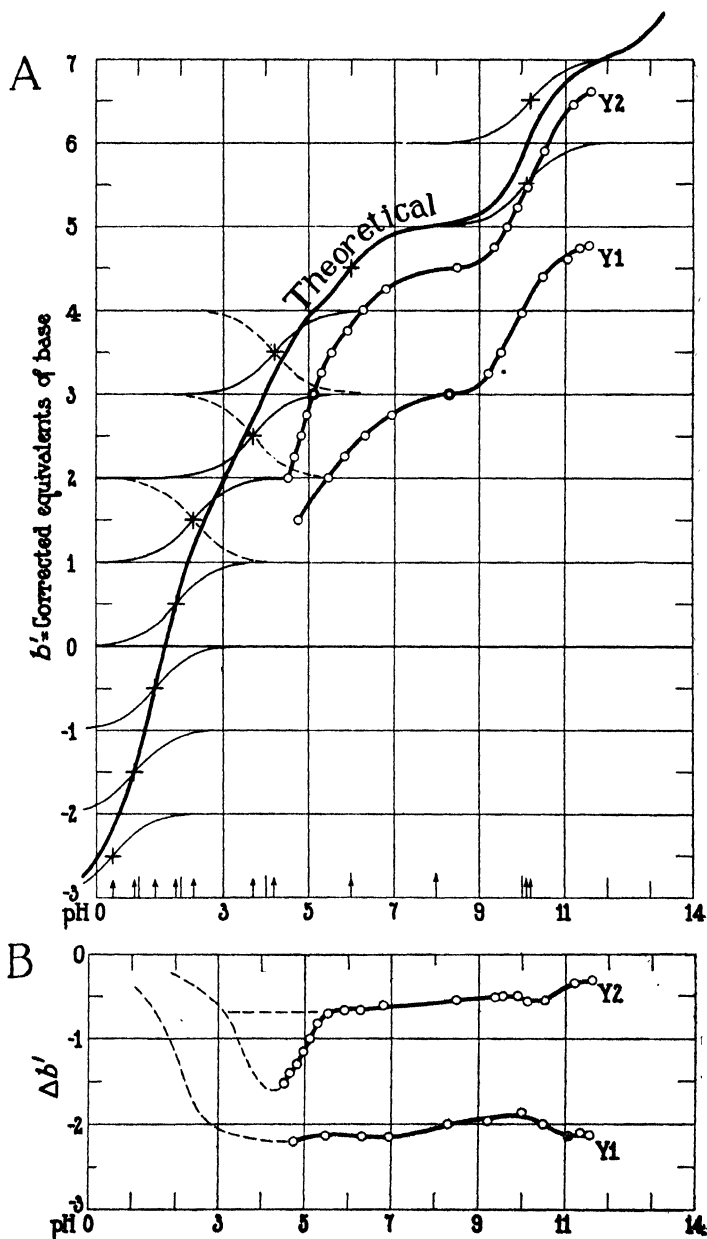


FIG. 1. A, theoretical curve of plant or animal nucleic acid with the experimental titration curves of two samples of yeast nucleic acid. B, corresponding comparison curves.

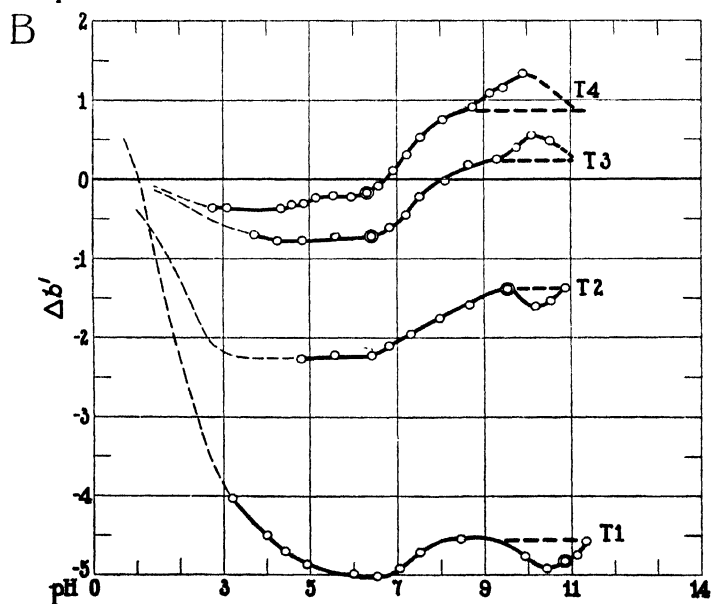
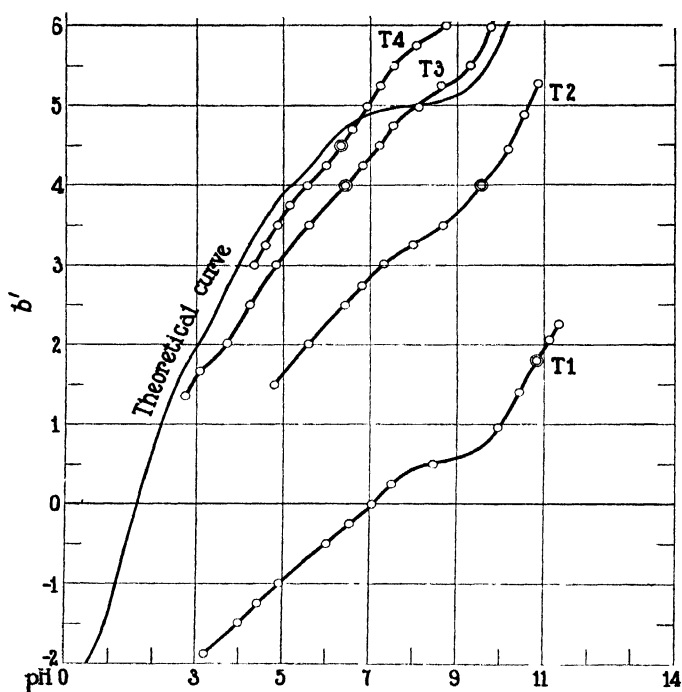


FIG. 2. *A*, experimental titration curves; *B*, comparison curves; of *thymus nucleic acid*.

no indication of an extra group with a constant equal to about 7.0. (Note horizontal curve from pH 6 to 9 in Fig. 1, B.) This shows that there is only one secondary phosphoric group, and hence, that the structure proposed by Levene is correct.<sup>6</sup>

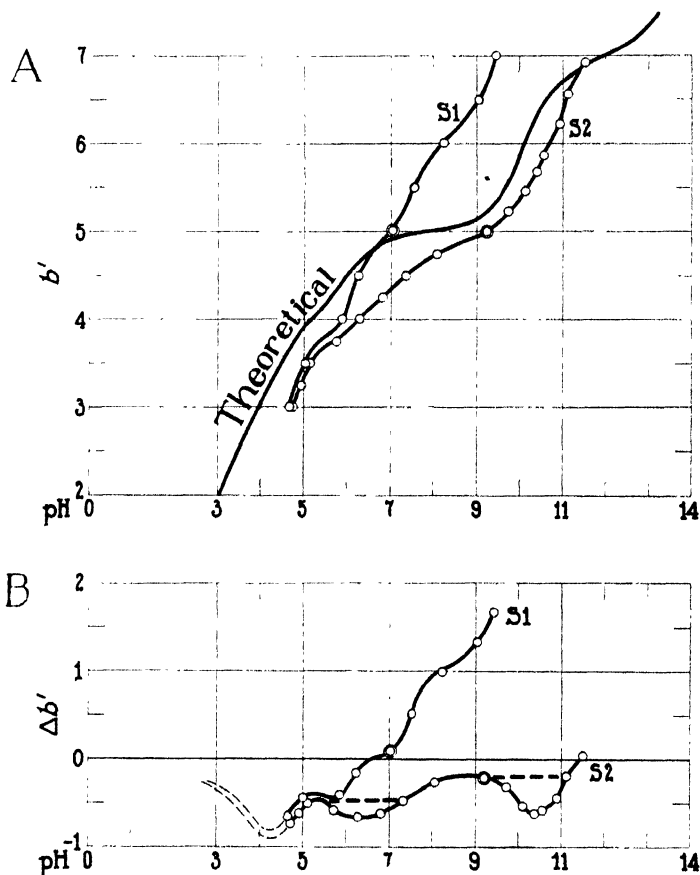


FIG. 3. A, experimental titration curves; B, comparison curves; of *spleen nucleic acid*.

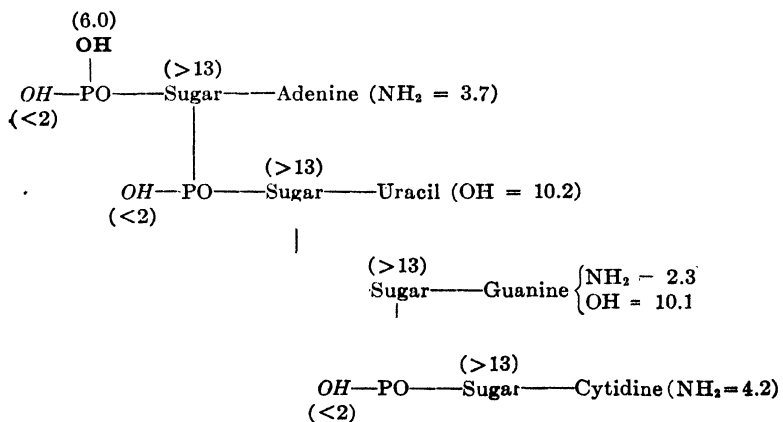
<sup>6</sup> It might be argued that Jones' structure is correct, thereby giving two phosphoric groups each having two ionizable groups; but that one of these was bound as anhydride. In the first place, this is improbable. In the second place, this argument cannot hold in the case of Y2 since this has only  $\frac{1}{2}$  equivalent of anhydride (Table II, Column 14). Hence, nearly an equivalent should dissociate with a constant at 7.0 if Jones' structure were correct.

3. *Animal Nucleic Acid. A. Thymus.*—Four samples of thymus nucleic acid (Fig. 2) show  $\frac{2}{3}$  to 5 equivalents of anhydride (at pH 6) and  $\frac{1}{2}$  to 1 equivalent of dissociation at  $pG' =$  about 7.5. Since chemical evidence has already proved the structure of animal nucleic acid, and since these materials were not as pure as the yeast nucleic acid, it may be concluded that the pure material does not dissociate at  $pG' = 7.5$ . This conclusion is substantiated by the results with spleen nucleic acid.

*B. Spleen.*—Two samples of spleen nucleic acid gave the values in Fig. 3. Of these S1 was an old, not very pure sample. It shows very marked deviation from the theoretical curve and definite dissociation of  $pG' = 7.5$ . Further purification of this sample gave S2 which approaches more nearly the theoretical curve and has only  $\frac{1}{2}$  equivalent of dissociation at  $pG' = 7.5$ . We may conclude that the purer the material, the more nearly does it approach the titration curve predicted from Levene's structure.

4. *Structural Differences.*—The drops and rises in the curves at about pH 10 represent slight deviations in the numerical values of  $pG'_9$  and  $pG'_{10}$  from those in Table I. This is due to spatial differences, not to chemical differences, and is related to the amount of anhydride.

A simplified structure of yeast nucleic acid is therefore as follows, where the numbers indicate the  $pG'$  values of the various ionizable groups:<sup>7</sup>



<sup>7</sup>It is to be understood that a given  $pG'$  value does not, strictly speaking, correspond to a given group, although some particular group may predominate in determining its numerical value. However, in nucleic acid the  $pG'$  values above 2 correspond, for all practical purposes, to given chemical groups.

*Theoretical Titration Constants of Nucleic Acid.*

From the values of the dissociation constants of the four nucleotides<sup>1</sup> we may estimate the titration constants<sup>2</sup> by means of formulas derived in another article.<sup>3</sup>

The *intrinsic* constants should be very nearly equal to those of the respective groups in the nucleotides, except that there will be only one secondary phosphoric group (according to Levene's structure). The *dissociation* constants depend upon the mutual influence between the groups (and upon the number of groups). The *titration* constants are directly dependent upon the relative numerical values of the dissociation constants.

However, as was pointed out,<sup>3</sup> when groups are sufficiently separated in space, the *titration* constants are equal to the respective *intrinsic* constants. In like manner the *titration* constants of nucleic acid will be identical with the *dissociation* constants of the nucleotides, except in so far as the electrostatic forces between the four nucleotide radicals affect the various groups. With this in mind, we may draw the following conclusions.

1. The four "bases" in nucleic acid are probably about 12 Å apart. Hence we may take the values of their amino groups as being about the same as in the nucleotides. (See values for  $pG_6'$ ,  $pG_6'$ , and  $pG_7'$  in Table I.)

2. It must be observed, however, that the molecule has five negatively charged groups, and no positively charged groups in the range of the ionization of the hydroxyl groups in uracil and guanine. In the nucleotides there are two negatively charged groups in this range. The effect of the three additional groups will be about  $3 \times \frac{3.0}{12} = 0.75$  which gives the values of  $pG_6'$  and  $pG_{10}'$  in Table I. The distortional work is neglected here since it should be quite small.

3. The groups in cytidine phosphoric acid and uridine phosphoric acid which ionize at 13.2 and 13.9, respectively, were referred to as hydroxyl groups of the bases but are now known to be due to ionization of the sugar groups. We have assigned values  $>13$  to each sugar group in nucleic acid. Their exact value does not matter since we do not titrate up into that range.

4. The four primary phosphoric groups should have intrinsic constants about equal to the first dissociation constants of the

nucleotides (0.7 to 1.0). It would be very complicated to attempt to calculate accurately the dissociation and the titration constants of these groups. However, we may estimate that the dissociation constants should be roughly about  $-0.2$ ,  $0.4$ ,  $1.4$ , and  $2.0$ , respectively. This gives for the values of the titration constants about  $-0.1$ ,  $0.5$ ,  $1.3$ , and  $1.9$ . The values plotted in the figures are  $0.3$ ,  $0.9$ ,  $1.3$ , and  $1.9$  which is close enough since we cannot titrate in that range.

5. Last of all, we come to the secondary phosphoric ionization. According to the structure of Levene, there should be only one such group. We may assign to it the same value as in the nucleotides ( $6.0$ ).

If Jones' structure of yeast nucleic acid were correct, there would be another such group. In this case, the value of the first constant ( $6.0$ ) should not be appreciably affected (since the molecule would be sufficiently rigid, due to the charges on the ionized groups, to reduce the distortional work to a minimum), but the value of the second constant would depend upon electrostatic forms between these two groups (practically regardless of all other groups). This, in turn, would depend upon the relative positions of these two groups in space which is a difficult factor to estimate in this molecule. We can say merely that this extra group would have a titration constant with a  $pG'$  value between  $6.0$  and  $7.5$ , probably not higher<sup>8</sup> than  $7.0$ .

We attempted to titrate cytidine diphosphoric acid in order to get the correct values for these constants. However, the material poisoned several electrodes and gave poor results. It probably has secondary phosphoric constants at about  $6.0$  and  $7.5$ . The impurities which buffer in that range (in the samples of animal nucleic acid) also have constants at about  $7.5$ .

#### *Interpretation of the Curves.*

In Table II the comparison curves in Figs. 1,*B*, 2,*B*, and 3,*B* are interpreted. The blank spaces in Columns 5 to 8 indicate that the comparison curve is horizontal and that the groups

<sup>8</sup> If the value is more than  $7.0$ , the distortional work could not be neglected and the value of the first constant would be less than  $6.0$ .

TABLE II.  
*Interpretation of Comparison Curves.*

Sample of nucleic acid.	Symbol.	Analyses.		Extra groups (+) or absence of groups (-).					Deviation in pG' values.				Anhydride.		
		No. (2)	Nitrogen. (3)	Phosphorus. (4)	NH <sub>2</sub> groups. (5)	pH 5-7 (6)	Secondary phosphoric group. (7)	Extra secondary phosphoric group. (8)	OH groups. (9)	NH <sub>2</sub> groups. (10)	pH 5-7 (11)	pH 7-9 (12)	OH groups. (13)	Observed. (14)	Corrected for P content.

Columns 3 and 4 give the equivalents of nitrogen and phosphorus in excess (+) or in deficiency (-), by analysis. Columns 5 to 8 give the equivalents of extra groups (+) or deficiency (-), as indicated by the comparison curves in the various pH ranges.

Columns 9 to 12 give the deviations in pG' values of the various groups, from the values given in Table I.

Column 13 gives the number of primary phosphoric groups bound as anhydride, as observed from the comparison curve at pH 6. Column 14 gives this value corrected for the phosphorous content.

Blank spaces indicate that the observed results correspond with the theoretical values.



ionizing in the respective pH ranges are those indicated in Table I. The numbers indicate the equivalents of additional (or deficient) groups. T1 shows a deficiency of at least one amino group (in agreement with the analysis). All the samples of animal nucleic acid show varying quantities of an extra secondary phosphoric group, apparently due to impurity. The  $pG'$  value of this group is 7.5 instead of 7.0. Hence, the secondary phosphoric groups in the impurity are closer together in space than would correspond to Jones' structure.

The blank spaces in Columns 9 to 12 (Table II) show that the predicted constants (given in Table I) are correct in the respective ranges. The numbers indicate the deviation of the observed values from the theoretical ones. This deviation is indicated by a drop or rise such as in the comparison curves about pH 10 and described in the preceding article.

#### EXPERIMENTAL.

0.001 mol of each sample of nucleic acid was treated with normal NaOH until dissolved. The quantity of NaOH required is indicated by an asterisk in Tables III to V and by a larger circle in Fig. 1 to 3. This solution was made up to 100 cc. in a volumetric flask (concentration equal to 0.01 molar). 5 cc. samples of this mother solution were treated with various quantities of 0.1 molar NaOH or HCl, and made up to 10 cc. (concentration equal to 0.005 molar). The pH of each solution was determined in a water-jacketed hydrogen electrode<sup>9</sup> at 25°C. The pH of 0.1 normal HCl was taken as 1.090, and the liquid junction potential with the saturated KCl bridge was assumed constant.

The results are given in Tables III to V. The values of  $b'$  are calculated from the approximate formula

$$b' = \frac{b - a}{c} + \frac{H - OH}{c} \quad (35)$$

where  $a$ ,  $b$ , and  $c$  are the *concentrations* (of strong acid, of strong

<sup>9</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1923, xiv, 2503.

base, and of substance, respectively) while  $H$  and  $OH$  are activities. This equation comes from the exact expression:

$$b' = b - a + h - oh \quad (34)$$

where  $h$  and  $oh$  are concentrations. When  $b'$  is approximately equal to  $\frac{b}{c}$  the values are omitted. The numbers of the equations correspond to those in a previous article.<sup>2</sup>

The values of  $\Delta b'$ , in the tables, refer to the difference at a given pH between the experimental value of  $b'$  and the calculated one ( $b'_t$  theoretical curve),  $\Delta b' = b' - b'_t$ . These are obtained graphically from the plots (Fig. 1 to 3).

TABLE III.  
*Yeast Nucleic Acid.*

$\frac{b}{c}$	Y1			Y2		
	pH	$b' = \frac{b-a}{c} + \frac{H-OH}{c}$	$\Delta b' = b' - b'_t$	pH	$b' = \frac{b}{c} + \frac{H-OH}{c}$	$\Delta b' = b' - b'_t$
1.50	(4.75)		-2.20			
2.00	5.38		-2.13	4.52	2.01	-1.52
2.25	5.86		-2.12	4.67		-1.40
2.50	6.33		-2.15	4.82		-1.30
2.75	6.96		-2.14	4.96		-1.14
3.00	8.31	3.00*	-2.00	5.12	3.00*	-1.00
3.25	9.22		-1.96	5.30		-0.82
3.50	9.50	3.49	-2.53	5.55		-0.70
3.75				5.92		-0.67
4.00	10.00	3.97	-1.85	6.29		-0.66
4.25	11.36			6.81		-0.61
4.50	10.48	4.40	-2.00	8.49		-0.54
4.75				9.36	4.74	-0.51
5.00	11.07	4.60	-2.14	9.65	4.98	-0.50
5.25				9.90	5.22	-0.50
5.50	11.36	4.74	-2.10	10.13	5.46	-0.56
6.00	11.58	4.76	-2.13	10.53	5.89	-0.75
7.00				11.22	6.45	-0.35
8.00				11.62	6.62	-0.32

\* Quantity of NaOH required to dissolve sample.

TABLE IV.  
*Thymus Nucleic Acid.*

$\frac{b}{c}$	T1			T2			T3			T4		
	pH	b'	$\Delta b'$	pH	b'	$\Delta b'$	pH	b'	$\Delta b'$	pH	b'	$\Delta b'$
-2.00	3.22	-1.88	-4.03									
-1.50	4.00	-1.48	-4.50									
-1.25	4.43	-1.24	-4.70									
-1.00	4.93		-4.87									
-0.50	6.00		-4.98									
-0.25	6.54		-5.02									
0	7.07		-4.92									
+0.25	7.51		-4.72									
+0.50	8.47		-4.54									
+1.00	9.93	0.97	-4.76				2.76	(1.35)	(-0.36)			
+1.50	10.43	1.41	-4.91	4.81		-2.26	3.09	(1.66)	(-0.36)			
+2.00	10.84	1.81*	-4.82	5.59		-2.21	3.71	2.02	-0.70			
+2.50	11.12	2.06	-4.74	6.43		-2.22	4.25		-0.77			
+2.75				6.82		-2.10						
+3.00	11.35	2.26	-4.57	7.33		-1.95	4.83		-0.77	4.35		-0.36
+3.25				8.00		-1.75				4.60		-0.32
+3.50				8.68		-1.58	5.60		-0.72	4.88		-0.30
+3.75										5.16		-0.23
+4.00				9.56	4.00*	-1.38	6.43	4.00*	-0.72	5.56		-0.20
+4.25							6.84		-0.61	5.98		-0.22
+4.50				10.20	4.45	-1.60	7.21		-0.44	6.32	4.50*	-0.17
+4.75							7.53		-0.22	6.60		-0.08

+5.00	10.54	4.88	-1.53	8.11	-0.02	6.93	+0.12
+5.25				8.66	(+0.18)	7.23	+0.31
+5.50	10.88	5.27	-1.36	9.31	+0.25	7.56	+0.52
+5.75				9.77		8.07	+0.75
+6.00				9.77	+0.40	8.75	+0.92
+6.25						9.14	+1.08
+6.50				10.10	+0.55	9.47	+1.15
+7.00				10.51	+0.48	9.90	+1.29
					6.46	6.49	
					6.89		

\* Quantity of NaOH required to dissolve sample.

TABLE V.  
*Spleen Nucleic Acid.*

Both samples gave unreliable pH readings.

$\frac{b}{c}$	S1			S2		
	pH	'	$\Delta b'$	pH	$b'$	$\Delta b'$
3.00	4.68		-0.65	4.73		-0.73
3.25				4.94		-0.62
3.50	5.02		-0.44	5.13		-0.50
3.75				5.74		-0.58
4.00	5.88		-0.41	6.30		-0.66
4.25				6.81		-0.62
4.50	6.27		-0.16	7.35		-0.47
4.75				8.08		-0.26
5.00	7.03	5.00*	+0.09	9.22	5.00*	-0.22
5.25				9.73	5.23	-0.31
5.50	7.53		+0.52	10.12	5.46	-0.54
5.75				10.40	5.68	-0.62
6.00	8.23		+0.98	10.58	5.87	-0.59
6.50	9.07		+1.33	10.91	6.23	-0.45
7.00	9.44		+1.67	11.11	6.57	-0.20
8.00				11.51	6.93	+0.03

\* Quantity of NaOH required to dissolve sample.

#### SUMMARY.

The titration constants of nucleic acid (see Table I) have been calculated from the dissociation constants of the nucleotides on the basis of relations between structure and dissociation. From these constants the theoretical titration curve has been drawn for plant or animal nucleic acid in which the four nucleotides are *all* linked by *ester* linkages (between phosphoric and sugar groups).

The experimental curves of various samples of nucleic acid have also been drawn and are interpreted by the comparison curve method described in the previous article.

Two samples of *yeast* nucleic acid have constants practically identical with the theoretical constants between pH 5.5 and pH 11.5. *This supports the structure proposed by Levene.* If there were an *ether* linkage, as claimed by Jones, there would be an extra secondary phosphoric group with a constant of about 7.0. There is no indication of such a group.

Animal nucleic acid has been shown, on chemical evidence, to have *ester* linkages between all the nucleotides. Four samples of *thymus* nucleic acid and two of *spleen* nucleic acid correspond with the theoretical curve except that various quantities of an extra group with a constant of about 7.5 are found. This is probably due to impurity. The amount of this group in a sample of spleen nucleic acid was reduced on further purification from 1.5 to 0.25 equivalent.

All samples showed deficiencies in primary phosphoric groups. Evidently these were bound as anhydride. The extent of this effect varied considerably between the different samples.

Only slight deviations were observed between the calculated and observed titration constants. The latter varied slightly among the different samples and were related to the amount of anhydride.



## DIACETONE GLUCOSE.

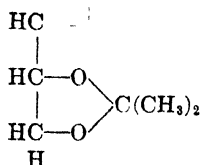
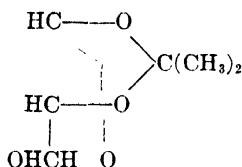
### III. METHYLATED METHYL GLUCOSIDES PREPARED FROM MONOACETONE GLUCOSE.

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The structure of diacetone glucose may be regarded as definitely established by the recent work of Levene and Meyer,<sup>1</sup> of Freudenberg and Doser,<sup>2</sup> of Freudenberg and Brauns,<sup>3</sup> and of Levene and Simms.<sup>4</sup> The latter authors have proved that the trimethyl-glucose obtained from monoacetone glucose has a five-membered ring structure. Thus, to diacetone glucose the following structure may be attributed:



<sup>1</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liv, 805; 1924, lx, 173.

<sup>2</sup> Freudenberg, K., and Doser, A., *Ber. chem. Ges.*, 1923, lvi, 1244.

<sup>3</sup> Freudenberg, K., and Brauns, F., *Ber. chem. Ges.*, 1922, lv, 3243.

<sup>4</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxxv, 31.



However, it was noted by Irvine and his coworkers,<sup>5</sup> who were the first to prepare the methylated derivatives of diacetone glucose, that the trimethylglucose prepared from monoacetone glucose behaved abnormally. In a general way, it resembled the  $\gamma$ -glucosides prepared by Fischer. Irvine therefore classified the acetone glucose among the  $\gamma$ -sugars.

Two distinctive peculiarities of trimethyl monoacetone glucose have always been emphasized. One is its levorotation and the other its power to reduce Fehling's solution in the cold. The observations recently made by us on pentamethylglucose caused us to undertake anew the study of the trimethylglucose prepared from monoacetone glucose. We observed that the pentamethylglucose did not possess as high a degree of reactivity as was attributed to the above trimethylglucose. Furthermore, whereas the sugar itself was levorotatory, its diacetal was dextrorotatory.

Now we have prepared 3,5,6-trimethylglucose from monoacetone glucose, and from this substance we obtained dextrorotatory or levorotatory products depending upon the conditions of the experiments.

#### *First Set of Experiments.*

In these experiments the monoacetone derivatives were hydrolyzed with dilute alcohol containing 0.5 per cent of hydrochloric acid. A levorotatory 3,5,6-trimethylglucose was obtained ( $[\alpha]_D = -8^\circ$  to  $-11^\circ$ ). When this product was converted into the glucoside the latter was dextrorotatory ( $[\alpha]_D = +21.4^\circ$ ). This glucoside was then converted into the 2,3,5,6-tetramethyl methyl glucoside which also was dextrorotatory ( $[\alpha]_D = +29.2^\circ$ ).

When the monoacetone trimethylglucose was converted into the 3,5,6-trimethyl methyl glucoside by digestion with methyl alcohol containing 0.5 per cent of hydrogen chloride gas, the product could be fractionated into two fractions; one, the lower boiling, was dextrorotatory ( $[\alpha]_D = +75^\circ$ ) and the other, the higher boiling fraction, was levorotatory ( $[\alpha]_D = -47^\circ$ ). These two fractions were combined and further methylated and the 2,3,

<sup>5</sup> Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 574. Irvine, J. C., Fyffe, A. W., and Hogg, T. P., *J. Chem. Soc.*, 1915, cvii, 524. Irvine, J. C., and Macdonald, J. L. H., *J. Chem. Soc.*, 1915, cvii, 1709. Irvine, J. C., and Patterson, J., *J. Chem. Soc.*, 1922, cxxi, 2160.

5,6-tetramethyl methyl glucoside obtained from it was levorotatory ( $[\alpha]_D = -12.4^\circ$ ).

The methylated sugars obtained on hydrolysis of these glucosides did not reduce Fehling's solution in the cold.

A peculiarity of all the derivatives of this class was the following. On heating with orcinol and hydrochloric acid they all condensed into a very heavy deep bluish pigment. The latter had the absorption bands characteristic for the analogous product obtained on treatment of pentoses with orcinol.

### *Second Set of Experiments.*

In this set of experiments the trimethylglucose obtained as above was distilled at 0.3 mm. pressure. The substance was levorotatory ( $[\alpha]_D = -37^\circ$ ), as described by Irvine and Patterson, and generally possessed the properties described by the latter workers. It differed in one respect. On conversion into its glucoside it gave two fractions. One, the lower boiling, was dextrorotatory ( $[\alpha]_D = +50^\circ$  (average)) and the other, the higher boiling, levorotatory ( $[\alpha]_D = -17^\circ$  to  $-50^\circ$ ). Each product was further methylated into two 2,3,5,6-tetramethyl methyl glucosides. The product obtained from the first fraction was dextrorotatory ( $[\alpha]_D = +63^\circ$ ), from the second, levorotatory ( $[\alpha]_D = -54^\circ$ ).

All these substances gave a strong orcinol test as did the substances obtained from the first set of experiments.

### DISCUSSION.

Of the two properties attributed to the  $\gamma$ -methylated sugars<sup>1</sup> one, namely, the power to reduce Fehling's solution in the cold, is undoubtedly due to an impurity, and the other, the levorotation, is not a constant property of the methylated sugars derived from diacetone glucose. As yet we are unable to decide whether the dextro- and levorotatory substances are  $\alpha$ - and  $\beta$ -isomers or ring isomers.

The method of recognizing the ring structure first recommended by Levene and Simms and later employed by Charlton, Haworth and Peat<sup>2</sup> will be applied to determine the character of the isomerism.

<sup>1</sup> Charlton, W., Haworth, W. N., and Peat, S., *J. Chem. Soc.*, 1926, cxxviii, 89.

As compared with pentamethylglucose, the aldehydic form, the above  $\gamma$ -sugars were found less reactive. Whereas the pentamethylglucose is readily converted into its diacetal on the addition of a little hydrogen chloride to its methyl alcoholic solution at room temperature, the trimethylglucoses require for conversion into the corresponding glucosides higher temperatures.

The rate of hydrolysis of the 2,3,5,6-tetramethyl methyl glucosides was found higher than that of 2,3,4,6-tetramethyl methyl glucoside.

Since publication of newer views on the ring structure in the common forms of tetramethylglucoses, the methylated sugars discussed in a previous paper<sup>7</sup> and the tables on page 537 of that paper will have to be revised as shown in Tables I and II.<sup>8</sup>

TABLE I.  
*Specific and Molecular Rotation of Methylated Gluconic Acids.*

Methylated derivative.	Solution in 1 equivalent NaOH.		The same solution neutralized.	
	$[\alpha]_D$	$[M]_D$	$[\alpha]_D$	$[M]_D$
2,3-Dimethyl.....	+43.7	+97.46	+22.5	+50.17
3,5,6-Trimethyl.....	+24.0	+56.88	-6.35	-15.05
2,3,4-Trimethyl.....	+64.4	+152.62	+38.6	+101.48
2,3,4,6-Tetramethyl.....	+76.4	+191.76	+43.4	+108.93
2,3,4,5,6-Pentamethyl.....	+53.7	+141.30	+22.5	+59.62

TABLE II.  
*Change in the Numerical Value of Rotation Due to Substitution in Different Positions.*

Carbon atom No.	$[M]_D$
2	+29.3
3	+42.5
4	+55.2
5	-50.3
6	+39.1

<sup>7</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1925, lxxv, 535.

<sup>8</sup> On page 542 of the same paper the formulae for the specific rotations + 19.3° and + 23.5° should read

$$[\alpha]_D^{\circ} = \frac{+0.75^{\circ} \times 100}{2 \times 0.976} = +38.6^{\circ}. \quad [\alpha]_D^{30} = \frac{+0.92^{\circ} \times 100}{2 \times 0.976} = +47.0^{\circ}.$$

## EXPERIMENTAL.

*3,5,6-Trimethyl Monoacetone Glucose*.—Monoacetone glucose was methylated with dimethyl sulfate and sodium hydroxide as previously described. The syrup distilled at 115–118°C.,  $p = 0.6$  mm. (from a metal bath at 135°C.).

The analysis of an average sample was

0.1002 gm. substance:	0.0206 gm. CO <sub>2</sub> and 0.0776 gm. H <sub>2</sub> O.
0.1406 " " :	(Zeisel) 16.6 cc. 0.1 N AgNO <sub>3</sub> .
C <sub>12</sub> H <sub>22</sub> O <sub>6</sub> .	Calculated. C 54.96, H 8.47, (OCH <sub>3</sub> ) 35.5.
	Found. " 54.56, " 8.66, " 36.6.

The specific rotations of various samples varied between  $[\alpha]_D = -27.4^\circ$  and  $-29.7^\circ$ . The syrup does not reduce Fehling's solution without previous hydrolysis.

*Conversion of Trimethyl Monoacetone Glucose into Trimethyl Methyl Glucoside.*

10 gm. of trimethyl monoacetone glucose were dissolved in 100 cc. of dry methyl alcohol containing 0.5 per cent hydrogen chloride and heated in an autoclave for 18 hours at 100°C. The product was neutralized with sodium methylate. The solvent was removed under diminished pressure and the product taken up in a large volume of dry ether, filtered with the addition of a small amount of norit, and the ether removed under diminished pressure. The syrup was distilled and boiled between 100–130°C.,  $p = 0.4$  mm. This product was fractionated into: 1, boiling at 100–120°C. and 2, boiling at 125–130°C.,  $p = 0.4$  mm. Fraction 1 reduced Fehling's solution slightly on boiling whereas Fraction 2 did not. The two fractions analyzed as follows:

1.	0.0962 gm. substance:	0.1742 gm. CO <sub>2</sub> and 0.0740 gm. H <sub>2</sub> O.
2.	0.1064 " " :	0.1948 " CO <sub>2</sub> " 0.0812 " H <sub>2</sub> O.
1.	0.1264 " " :	(Zeisel) 21.70 cc. 0.1 N AgNO <sub>3</sub> .
2.	0.1267 " " :	" 21.10 " 0.1 " "
	C <sub>9</sub> H <sub>18</sub> O <sub>6</sub> .	Calculated. C 50.95, H 8.47, (OCH <sub>3</sub> ) 52.50.
		Found 1. " 49.38, " 8.60, " 53.22.
		2. " 49.92, " 8.54, " 51.62.

The specific rotations were found to be in methyl alcohol

$$1. \quad [\alpha]_D = \frac{+4.48^\circ \times 100}{1 \times 5.96} = +75^\circ.$$

$$2. \quad [\alpha]_D = \frac{-2.20^\circ \times 100}{1 \times 4.70} = -47^\circ.$$

*Conversion of Trimethyl Methyl Glucoside into Tetramethyl Methyl Glucoside.*

22 gm. of material prepared as described in the preceding paragraph but without fractionation were dissolved in 200 cc. of dry ether and treated with 4 gm. of metallic sodium. After the reaction had ceased the ether was removed on the steam bath using a fractionating column. A decided odor of a volatile aldehyde was noticed, but its nature for the present was not further established. The solvent was finally completely removed under diminished pressure, and 20 cc. of methyl iodide were added. After 4 hours sodium iodide had separated and the reaction was terminated by the addition of an excess of ether.

The syrup obtained after removal of the ether weighed 12 gm., distilled at  $112^\circ\text{C}$ .,  $p = 0.8$  mm., and had a specific rotation in water of

$$[\alpha]_D = \frac{-0.75^\circ \times 100}{1 \times 7.7} = -9.9^\circ.$$

On standing for a few days the syrup became slightly colored. It was therefore redistilled. Except for a slight residue all distilled at  $102^\circ\text{C}$ .,  $p = 0.3$  mm., and analyzed as follows:

0.0964 gm. substance: 0.1830 gm.  $\text{CO}_2$  and 0.0765 gm.  $\text{H}_2\text{O}$ .

0.1262 " " : (Zeisel) 25.5 cc. 0.1 N  $\text{AgNO}_3$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6$ . Calculated. C 52.80, H 8.89,  $(\text{OCH}_3)$  62.00.

Found. " 51.76, " 8.87, " 62.63.

The specific rotation in water was now

$$[\alpha]_D = \frac{-0.57^\circ \times 100}{1 \times 4.58} = -12.4^\circ.$$

It is soluble in alcohol, ether, and water and does not reduce Fehling's solution until hydrolyzed. The rate of hydrolysis will be found in Table III C.

*Hydrolysis of Trimethyl Monoacetone Glucose.*

This was accomplished according to the directions of Irvine and Scott. The resulting syrup was dried at 100° under high vacuum and analyzed as follows:

0.1042 gm. substance: 0.1864 gm. CO<sub>2</sub> and 0.0778 gm. H<sub>2</sub>O.  
 0.1288 " " : (Zeisel) 17.6 cc. 0.1 N AgNO<sub>3</sub>.  
 C<sub>9</sub>H<sub>11</sub>O<sub>6</sub>. Calculated. C 48.75, H 8.11, (OCH<sub>3</sub>) 41.9.  
 Found. " 48.77, " 8.35, " 42.3.

The specific rotation in methyl alcohol of several samples varied between

$$[\alpha]_D = -8.0^\circ \text{ and } [\alpha]_D = -11.4^\circ.$$

The substance did not reduce Fehling's solution in the cold, but did so on boiling. It gave a very strong orcinol reaction.

\* *Conversion of the "Non-Distilled" Trimethyl Glucose into a Dextro-Trimethyl Methyl Glucoside.*

45 gm. of the sugar dissolved in 400 cc. of dry methyl alcohol containing  $\frac{1}{4}$  per cent hydrogen chloride were heated in an autoclave for 20 hours at 100°C. The reaction product was only slightly colored. The acid was neutralized with sodium methylate and further treatment was as previously described. The syrup distilled at 135°C.,  $p = 0.6$  mm., and analyzed as follows:

0.0946 gm. substance: 0.1759 gm. CO<sub>2</sub> and 0.0730 gm. H<sub>2</sub>O.  
 0.1130 " " : (Zeisel) 19.1 cc. 0.1 N AgNO<sub>3</sub>.  
 C<sub>10</sub>H<sub>12</sub>O<sub>6</sub>. Calculated. C 50.95, H 8.47, (OCH<sub>3</sub>) 52.5.  
 Found. " 50.70, " 8.63, " 52.4.

The substance had the following rotations.

$$\text{In methyl alcohol: } [\alpha]_D = \frac{+0.70^\circ \times 100}{1 \times 5.28} = +13.3^\circ.$$

$$\text{In water: } [\alpha]_D = \frac{+0.60^\circ \times 100}{1 \times 2.8} = +21.4^\circ.$$

The syrup is soluble in alcohol, ether, and water. It does not reduce Fehling's solution until after hydrolysis. The glucoside is very sensitive to acids. Heating in boiling water with 0.2 N HCl gives maximum reduction to Fehling's solution in 40 minutes. Longer heating is accompanied by destruction of the sugar.

*Conversion of the Above Dextro-Trimethyl Methyl Glucoside into a Dextro-Tetramethyl Methyl Glucoside.*

This was accomplished by the method of Freudenberg with sodium and methyl iodide as previously described.

The syrup distilled at 105°C.,  $p = 0.3$  mm., and analyzed as follows:

0.1001 gm. substance: 0.1918 gm. CO<sub>2</sub> and 0.0806 gm. H<sub>2</sub>O.

0.1368 " " : (Zeisel) 27.0 cc. 0.1 N AgNO<sub>3</sub>.

C<sub>11</sub>H<sub>22</sub>O<sub>6</sub>. Calculated. C 52.80, H 8.80, (OCH<sub>3</sub>) 62.00.

Found. " 52.25, " 8.94, " 61.18.

Its specific rotation in water was

$$[\alpha]_D = \frac{+1.05^\circ \times 100}{1 \times 3.6} = +29.2^\circ.$$

It did not reduce Fehling's solution until after hydrolysis. The rate of hydrolysis will be found in Table III B.

*Distillation of Trimethyl Glucose.*

The syrup obtained by the hydrolysis of trimethyl monoacetone glucose distills in part at 150°C.,  $p = 0.3$  mm. The analysis of one of the preparations was: C 50.13, H 8.59, (OCH<sub>3</sub>) 44.39. In methyl alcohol the specific rotation of several preparations immediately on solution varied between  $[\alpha]_D = -34^\circ$  and  $[\alpha]_D = -41^\circ$ . In one instance in which the rotation was observed for 24 hours it was initially  $\alpha = -1.4^\circ$  ( $c = 3.36$ ,  $l = 1$ ) and in 27 minutes became constant at  $\alpha = -0.75^\circ$ . On addition of  $\frac{1}{4}$  per cent hydrogen chloride without heating, the rotation which was  $-1.4^\circ$  became  $-1.2^\circ$ , and on heating at 100° for 1 hour the

rotation became  $\alpha = +0.10^\circ$ . Further heating produced no change in rotation. The distilled product differs from the non-distilled sugar in that it instantaneously reduces Fehling's solution in the cold.

*Two Trimethyl Methyl Glucosides from the Distilled Product.*

The distilled product, dissolved in 10 volumes of methyl alcohol containing  $\frac{1}{4}$  per cent hydrogen chloride, was heated at  $100^\circ$  for 18 hours. The reaction product was always deeply colored. Further treatment was similar to that previously described. The syrup was fractionated and boiled at:

1.  $110\text{--}115^\circ\text{C.}$ ,  $p = 0.4$  mm.
2.  $130^\circ\text{C.}$ ,  $p = 0.2$  mm.

The specific rotations of several preparations were as follows:

- For fraction 1.  $[\alpha]_D = +50^\circ$ ,  $+59.8^\circ$ , and  $+30^\circ$ .  
 " " 2.  $[\alpha]_D = -30^\circ$ ,  $-54^\circ$ , "  $-17^\circ$ .

None of these preparations reduced Fehling's solution in the cold, but all did so on warming.

The two fractions analyzed as follows:

1. 0.1030 gm. substance	0.1930 gm. $\text{CO}_2$ and 0.0786 gm. $\text{H}_2\text{O}$ .
0.1314 " "	(Zeisel) 22.7 cc. 0.1 N $\text{AgNO}_3$ .
2. 0.1056 " "	0.1936 gm. $\text{CO}_2$ and 0.0812 gm. $\text{H}_2\text{O}$ .
0.1376 " "	(Zeisel) 23.2 cc. 0.1 N $\text{AgNO}_3$ .
$\text{C}_{10}\text{H}_{20}\text{O}_6$ .	Calculated. C 50.95, H 8.80, $(\text{OCH}_3)$ 52.50.
	Found 1. " 51.08, " 8.53, " 52.03.
	2. " 49.99, " 8.60, " 52.27.



*Further Methylation of the Preceding Two Sugars to Tetramethyl Methyl Glucosides.*

The two products mentioned in the preceding paragraph were further methylated separately by the Freudenberg process, as described above. The action of sodium on Fraction 1 was more violent and produced a darker colored reaction product, and a heavier precipitate on the addition of ether.

The syrup obtained from Fraction 1 boiled at 98–100°C.,  $p = 0.3$  mm., and analyzed as follows:

0.0897 gm. substance: 0.1696 gm.  $\text{CO}_2$  and 0.0698 gm.  $\text{H}_2\text{O}$ .

0.1120 " " : (Zeisel) 22.2 cc. 0.1 N  $\text{AgNO}_3$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6$ . Calculated. C 52.80, H 8.80,  $(\text{OCH}_3)$  62.00.

Found. " 52.12, " 8.85, " 61.40.

The specific rotation in water was

$$[\alpha]_D = \frac{+3.20^\circ \times 100}{1 \times 5.064} + 63^\circ.$$

Another preparation had a rotation of  $[\alpha]_D = +30^\circ$ . It did not reduce Fehling's solution, even on boiling, until hydrolyzed. However, it gave a very strong orcinic test. The rate of hydrolysis will be found in Table III D.

The syrup obtained from Fraction 2 distilled at 110°C.,  $p = 0.5$  mm., and analyzed as follows:

0.0962 gm. substance: 0.1844 gm.  $\text{CO}_2$  and 0.0752 gm.  $\text{H}_2\text{O}$ .

0.1226 " " : (Zeisel) 24.6 cc. 0.1 N  $\text{AgNO}_3$ .

$\text{C}_{11}\text{H}_{22}\text{O}_6$ . Calculated. C 52.80, H 8.80,  $(\text{OCH}_3)$  62.00.

Found. " 52.26, " 8.74, " 62.18.

It had the following rotation in water:

$$[\alpha]_D = \frac{-2.57^\circ \times 100}{1 \times 4.69} = -54.9^\circ.$$

Another preparation had a rotation of  $[\alpha]_D = -39^\circ$ .

It also did not reduce Fehling's solution on boiling until after hydrolysis and gave a strong orcinol test. The rate of hydrolysis will be found in Table III E.

TABLE III.  
*Rate of Hydrolysis in 2 Per Cent HCl at 100°C.*

Time.	Reduction to Lehman-Maquenne calculated in per cent.				
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>
<i>min.</i>					
30	11.2	91.0	57.5	117.0	103.0
60	20.6	66.5	82.0	75.0	75.0
120	28.6				
240	65.5				

*A* is the rate of hydrolysis of 2,3,5-trimethyl methyl glucoside. In *B*, *D*, and *E* hydrolysis reached its maximum in 30 minutes. The concentrations in all experiments were approximately 1.0 per cent.

The reductions are calculated on the basis of values given in the paper by Sobotka, H., *J. Biol. Chem.*, 1926, lxi, 267.



## SUBSTITUTION BY HALOGEN OF THE HYDROXYL IN SECONDARY ALCOHOLS.\*

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In the previous communications on this subject, representative members of the following classes of secondary carbinols were discussed: (1) those in which two normal alkyl radicles are attached to the asymmetric carbon atom; (2) those in which one radicle contains a secondary carbon atom; (3) those in which one radicle is phenyl and the second benzyl. In the present paper results are reported on the halogenation of ethylphenyl carbinol, of two of its higher normal chain homologues, and also of isopropylphenyl carbinol, in which one radicle contains a secondary carbon atom and the second is aromatic. Methylphenyl carbinol has already been investigated by McKenzie and Clough.<sup>1</sup> The observation on that alcohol was the first which demonstrated the occurrence of Walden inversion in the series of carbinols.

The second member of this series, namely ethylphenyl carbinol, behaved exactly as methylphenyl carbinol. On treatment with hydrogen bromide a halide was obtained which rotated in the direction opposite to that of the parent alcohol, whereas treatment with thionyl chloride led to a substance rotating in the same direction.

The other carbinols, namely *n*-propylphenyl, isopropylphenyl, and *n*-butylphenyl, behaved differently from either the aliphatic or from the two lower members of the present series; that is, the halides obtained by either method rotated in the same direction as the parent carbinol.

\* This is the seventh paper of the series on Walden inversion.

<sup>1</sup> McKenzie, A., and Clough, G. W., *J. Chem. Soc.*, 1913, ciii, 698.

These observations bring out the fact that one and the same reagent may act differently even on as closely related substances as those discussed in this paper, producing Walden inversion in one member and not in another. We are inclined to believe that this difference in result is due to the differences in the degree of distortion of the tetrahedron of the asymmetric carbon atom brought about by the addition of the halogenating reagent. For the present, the data do not suffice to permit a quantitative analysis of the degree of distortion.

The table of the various halides is given below:

Carbinol.	Halogenat- ing agent.	$[\text{M}]_{\text{D}}$ carbinol.	$[\text{M}]_{\text{D}}$ halide.	$[\text{M}]_{\text{D}}$ carbinol maximum.
Methylphenyl carbinol.....	$\text{SOCl}_2$	+19.95°	+16.60°	-68.02°
Ethylphenyl carbinol.....	$\text{SOCl}_2$	-74.16°	-77.77°	-75.53°
“ “ .....	$\text{HBr}$	+47.36°	-3.00°	
<i>n</i> -Propylphenyl carbinol.....	$\text{SOCl}_2$	+35.08°	+45.29°	-85.81°
“ “ .....	$\text{HBr}$	+40.27°	+9.84°	
Isopropylphenyl carbinol.....	$\text{SOCl}_2$	-37.78°	-47.75°	+64.81°
“ “ .....	$\text{HBr}$	+70.54°	+58.55°	
<i>n</i> -Butylphenyl carbinol.....	$\text{SOCl}_2$	-6.57°	-24.50°	+61.24°
“ “ .....	$\text{PCl}_5$	-20.04°	-9.34°	

From this table it is seen that the molecular rotations of the halides prepared by means of thionyl chloride are with one exception numerically higher than those of the parent alcohols, whereas when the halides are obtained with the other reagents, they show racemization, of which the degree differs with each individual halide.

Of the five optically active carbinols employed in this work, two had been prepared and resolved before, namely methyl- and ethylphenyl carbinols, both by Pickard and Kenyon.<sup>2</sup> The remaining three had not been resolved before. Pickard and Kenyon had not succeeded in resolving ethylphenyl carbinol through the phthalic ester and had to resort to the succinic ester method. We were more fortunate with the usual phthalic ester method. However, we found that a certain condition is very essential for

<sup>2</sup> Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 45.

the resolution, namely the condition of the alkaloid. When an alkaloid is capable of crystallization in either the anhydrous or the hydrated form, it is important that conditions are maintained such that all through the fractionations, only one salt is formed, either the anhydrous or the hydrated. Sometimes with the hydrated form the dextro acid is the more insoluble and with the anhydrous form the enantiomorphous substance is the more insoluble. In the case of ethylphenyl carbinol anhydrous strychnine was used for resolution. It may be added that the active methyl- and ethylphenyl carbinols prepared by us had higher molecular rotations than those prepared by the other authors.

Another noteworthy peculiarity observed in the course of the preparation of the carbinols is that presented by *n*-propylphenyl carbinol. The strychnine salt of the phthalic ester of this carbinol showed a high tendency towards autoracemization even when kept in a desiccator at a temperature of about 15°C. The salt which gave a phthalate with an initial specific rotation of  $[\alpha]_D^{20} = -18.42^\circ$  was found after a few days standing to yield a phthalate with a specific rotation of  $[\alpha]_D^{20} = -8.09^\circ$ .

#### EXPERIMENTAL.

*Resolution of Methylphenyl Carbinol.*—To resolve the alcohol the general method of Pickard and Kenyon<sup>3</sup> was employed. The alcohol was converted into a half ester of phthalic acid by heating molecular proportions of the alcohol and phthalic anhydride at 100–105° for 2 hours under a return condenser. The phthalate when purified was converted into the brucine salt by treating it with 1 equivalent of brucine in acetone solution. At first, much difficulty was experienced in an endeavor to make the brucine salt crystallize, but when a little of the crystalline salt had been obtained, no further difficulty was met in starting crystallization. The brucine salt was then repeatedly recrystallized from acetone until the phthalate obtained from the salt no longer showed an increase in rotation. This was reached when the phthalate showed a rotation of

$$[\alpha]_D^{20} = \frac{+2.63^\circ \times 100}{1 \times 10.538} = +24.95^\circ.$$

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<sup>3</sup> Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1907, xci, 2058.

When the maximum activity was thus obtained, the brucine salt was decomposed with hydrochloric acid and the half ester was extracted with ether. The ester was then steam-distilled with  $2\frac{1}{2}$  mols of sodium hydroxide. The distillate was extracted with ether and dried over sodium sulfate. The ether was then removed and the residue distilled under reduced pressure (about 15 mm.). It boiled at 104–105°C., and showed a rotation of

$$[\alpha]_D^{20} = \frac{-4.98^\circ \times 100}{1 \times 9.077} = -54.86^\circ. \quad [M]_D^{20} = -68.02^\circ.$$

*Resolution of Ethylphenyl Carbinol.*—The carbinol was converted into the half ester of phthalic acid by heating 75 gm. of ethylphenyl carbinol with 77.5 gm. of phthalic anhydride at 110–115° for 2 hours. The reaction mixture was then poured into a solution of 54 gm. of sodium carbonate in 750 cc. of water. After allowing the solution to stand at room temperature for 2 hours it was extracted with ether to remove the unchanged carbinol. The mother liquor was then acidified with 25 per cent sulfuric acid. The phthalate which separated in the form of an oil was extracted with chloroform and dried over sodium sulfate. The chloroform was removed under reduced pressure. The residue solidified on cooling and stirring. 102 gm. of the phthalate were obtained. The phthalate was then dissolved in acetone and treated with 1 equivalent of strychnine alkaloid. On stirring and cooling the salt separated in a beautifully crystalline form. The salt was then repeatedly extracted with dry ethyl acetate at 36°C. In each case the salt was allowed to stand in the solution for 24 hours before it was filtered. This was repeated until the repetition of the process no longer increased the activity of the carbinol obtained from the phthalate.

The phthalate was isolated from the salt by treating the latter with hydrochloric acid and extracting the ester with ether. The most active phthalate showed a rotation of  $[\alpha]_D^{20} = -2.01^\circ$ . This was steam-distilled with  $2\frac{1}{2}$  mols of sodium hydroxide. The distillate was extracted with ether and the extract dried over sodium sulfate. The ether was removed and the residue fractionated under reduced pressure (about 15 mm.). The last fraction boiled at 119–120° and showed a rotation of

$$[\alpha]_D^{20} = \frac{-5.55^\circ \times 100}{1 \times 9.992} = -55.54^\circ. \quad [M]_D^{20} = -75.53^\circ.$$

The strychnine salt of the half ester showed a tendency to racemize on standing.

*Resolution of *n*-Propylphenyl Carbinol.*—100 gm. of *n*-propylphenyl carbinol were condensed with 100 gm. of phthalic anhydride by heating at 105–115°C. for 4 hours. The half ester was isolated and purified in the same way as the corresponding ethylphenyl carbinol derivative described above. The ester obtained weighed 120 gm. On cooling and stirring it solidified. The ester was converted into the strychnine salt by treating an acetone solution of the former with 1 equivalent of the alkaloid. The salt separated from the acetone solution very readily on cooling. It was repeatedly recrystallized from methyl alcohol until the phthalate when freed from the salt showed a rotation of

$$[\alpha]_D^{20} = \frac{-1.37^\circ \times 100}{1 \times 7.436} = -18.42^\circ.$$

\*After several days standing, the salt racemized to such an extent that the phthalate derived from it showed a rotation of only

$$[\alpha]_D^{20} = \frac{-1.55^\circ \times 100}{1 \times 19.140} = -8.09^\circ.$$

Lack of material prevented further recrystallization. The total supply of the salt on hand was, therefore, decomposed with hydrochloric acid, and the half ester obtained, which showed a rotation of  $[\alpha]_D^{20} = \frac{-1.43^\circ \times 100}{1 \times 16.63} = -8.59^\circ$ , was steam distilled with  $2\frac{1}{2}$

mols of sodium hydroxide. The distillate was extracted with ether, the extract dried over sodium sulfate, and then fractionated under reduced pressure (about 15 mm.). The carbinol boiled at 120–121°C. It was levorotatory.

$$[\alpha]_D^{20} = \frac{-2.96^\circ \times 100}{1 \times 5.174} = -57.21^\circ. \quad [M]_D^{20} = -85.81^\circ.$$

*Resolution of Isopropylphenyl Carbinol.*—150 gm. of isopropylphenyl carbinol were condensed with 150 gm. of phthalic anhydride by heating a mixture of the two for 4 hours at 115–118°C. The ester was isolated exactly in the same way as the corresponding *n*-propylphenyl derivative described above. The phthalate which crystallized on standing weighed 217 gm. It was converted into the strychnine salt in acetone solution, from which solution the



salt crystallized readily. The latter was then repeatedly recrystallized from methyl alcohol until the maximum activity was reached. The half ester then showed a rotation of

$$[\alpha]_D^{20} = \frac{-5.13^\circ \times 100}{1 \times 24.471} = -20.96^\circ.$$

The recrystallization filtrates from this levo-strychnine salt were decomposed with hydrochloric acid and the ester obtained was converted into the brucine salt in methylethyl ketone solution. After several recrystallizations from this solvent, a phthalate which gave a rotation of  $[\alpha]_D^{20} = \frac{+1.94^\circ \times 100}{1 \times 9.200} = +21.08^\circ$ , was obtained by decomposing the salt. This dextro-phthalate was steam-distilled with  $2\frac{1}{2}$  mols of sodium hydroxide. The distillate was extracted with ether, the extract dried over sodium sulfate, and finally fractionated under reduced pressure (about 15 mm.). The carbinol boiled at  $124\text{--}125^\circ\text{C.}$ , and showed a rotation of

$$[\alpha]_D^{20} = \frac{+3.24^\circ \times 100}{1 \times 6.797} = +47.66^\circ. \quad [M]_D^{20} = +64.81^\circ.$$

*Resolution of n-Butylphenyl Carbinol.*—75 gm. of *n*-butylphenyl carbinol were mixed with 67.5 gm. of phthalic anhydride and heated 4 hours at  $100\text{--}105^\circ\text{C.}$  The half ester was isolated as in the case of the corresponding derivatives in the previous experiments; 57 gm. of the half ester were obtained. This was converted into a cinchonidine salt in acetone solution, from which solution it crystallized readily. The salt was recrystallized from acetone until further recrystallizations no longer increased the rotation of the ester. This was reached when the ester showed a rotation of

$$[\alpha]_D^{20} = \frac{+2.92^\circ \times 100}{1 \times 21.930} = +13.31^\circ.$$

The ester was then steam-distilled with  $2\frac{1}{2}$  mols of sodium hydroxide. The distillate was extracted with ether, the extract dried over sodium sulfate, and fractionated under a pressure of about 15 mm. The carbinol boiled at  $121\text{--}122^\circ\text{C.}$ , and showed a rotation of

$$[\alpha]_D^{20} = \frac{+4.98^\circ \times 100}{1 \times 12.196} = +40.83^\circ. \quad [M]_D^{20} = +61.24^\circ.^4$$

<sup>4</sup> In view of the fact that the molecular rotation of this alcohol is lower than that of the preceding member of this series, it is desirable to make further efforts to obtain a sample with a higher rotation.

*Action of Thionyl Chloride on Dextro-Methylphenyl Carbinol.*—

12 gm. of dextro-methylphenyl carbinol ( $[\alpha]_D^{20} = \frac{+3.34^\circ \times 100}{1 \times 20.392} = +16.36^\circ$ ) were poured slowly with cooling into 54 gm. of thionyl chloride. After all the carbinol had been added, the reaction was brought to completion by heating on the steam bath under a return condenser for 15 minutes. The excess of thionyl chloride was then distilled off under reduced pressure. The residue was taken up with water and extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and was finally dried over sodium sulfate. When thoroughly dry, the ether was removed and the residue distilled under reduced pressure (about 15 mm.). It boiled at 80–81°C. and showed an optical rotation of

$$[\alpha]_D^{20} = \frac{+0.92^\circ \times 100}{1 \times 7.736} = +11.89^\circ. \quad [M]_D^{20} = +16.71^\circ \text{ (in ether).}$$

0.1308 gm. substance: 0.1350 gm. AgCl.

$C_8H_9Cl$ . Calculated. Cl 25.22.  
Found. " 25.53.

*Action of Thionyl Chloride on Levo-Ethylphenyl Carbinol.*—

This halogen derivative was prepared in a manner exactly analogous to the method described for the preparation of phenyl-methyl chloromethane. The phenylethyl carbinol used in this preparation showed a rotation of

$$[\alpha]_D^{20} = \frac{-4.67^\circ \times 100}{1 \times 8.564} = -54.53^\circ. \quad [M]_D^{20} = -74.16^\circ.$$

The chloride obtained was distilled under reduced pressure (about 15 mm.). It boiled at 86–90°C., and was found to be levorotatory.

$$[\alpha]_D^{20} = \frac{-6.71^\circ \times 100}{1 \times 13.327} = -50.34^\circ. \quad [M]_D^{20} = -77.77^\circ.$$

The optical activity of the carbinol, as well as that of the chloride, was determined in ether solution.

0.1482 gm. substance: 0.1358 gm. AgCl.

$C_8H_{11}Cl$ . Calculated. Cl 22.96.  
Found. " 22.66.

*Action of Hydrobromic Acid on Dextro-Ethylphenyl Carbinol.*—

5 gm. of dextro-ethylphenyl carbinol,  $[\alpha]_D^{20} = \frac{+2.88^\circ \times 100}{1 \times 8.274} = +34.83$  (in ether),  $[M]_D^{30} = +47.36^\circ$ , were saturated with dry hydrobromic acid gas at  $0^\circ\text{C}$ . The reaction took place almost immediately with the separation of water. When the reaction was completed, ice was added and the mixture was extracted with ether. The extract was washed free of hydrobromic acid and subsequently dried over sodium sulfate. The ether was then removed and the residue fractionated under a pressure of 0.04 mm., the boiling point being  $59\text{--}63^\circ\text{C}$ . The bromide was levorotatory.

$$[\alpha]_D^{30} = \frac{-0.28^\circ \times 100}{1 \times 18.570} = -1.51^\circ. \quad [M]_D^{30} = -3.00^\circ \text{ (in ether).}$$

0.1452 gm. substance: 0.1346 gm. AgBr.

$\text{C}_8\text{H}_{11}\text{Br}$ . Calculated. Br 40.16.

Found. " 39.45.

That the substance racemized considerably on distillation was seen from the fact that, before distillation, it showed a rotation of

$$[\alpha]_D^{20} = \frac{-0.59^\circ \times 100}{1 \times 10.392} = -5.66^\circ \text{ (in ether).}$$

*Action of Thionyl Chloride on Dextro-n-Propylphenyl Carbinol.*—

23 gm. of dextro-*n*-propylphenyl carbinol,  $[\alpha]_D^{20} = \frac{+2.25^\circ \times 100}{1 \times 9.616} = +23.39^\circ$ ,  $[M]_D^{20} = +35.08^\circ$  (in ether), were poured slowly with cooling into 92 gm. of thionyl chloride. The isolation and purification of the chloro body were carried out exactly as in the preparation of methylphenyl chloromethane. The substance distilled at  $107\text{--}110^\circ\text{C}$ ., under a pressure of about 15 mm., and rotated polarized light to the right.

$$[\alpha]_D^{20} = \frac{+1.36^\circ \times 100}{1 \times 5.058} = +26.88^\circ. \quad [M]_D^{20} = +45.29^\circ \text{ (in ether).}$$

0.1406 gm. substance: 0.1178 gm. AgCl.

$\text{C}_{10}\text{H}_{13}\text{Cl}$ . Calculated. Cl 21.06.

Found. " 20.72.

*Action of Hydrobromic Acid on Dextro-n-Propylphenyl Carbinol.*—

5 gm. of *n*-propylphenyl carbinol,  $[\alpha]_D^{20} = \frac{+2.64^\circ \times 100}{1 \times 9.831} = +26.85^\circ$  (in ether),  $[M]_D^{20} = +40.27^\circ$ , were saturated with dry

hydrobromic acid gas at 0°C. The reaction proceeded immediately with elimination of water, which collected at the bottom of the flask. When the reaction had come to an end, ice was added and the mixture was extracted with ether. The extract was washed free of hydrobromic acid and dried over sodium sulfate. The ether was then removed and the residue fractionated under a pressure of 0.04 mm., distilling under this pressure at 65–68°C. It rotated polarized light to the right.

$$[\alpha]_D^{20} = \frac{+0.41^\circ \times 100}{1 \times 8.8641} = +4.62^\circ. \quad [M]_D^{20} = +9.84^\circ.$$

0.1122 gm. substance: 0.0992 gm. AgBr.

$C_{10}H_{13}Br$ . Calculated. Br 37.51.  
Found. " 37.62.

*Action of Thionyl Chloride on Levo-Isopropylphenyl Carbinol.*—

15 gm. of levo-isopropylphenyl carbinol,  $[\alpha]_D^{20} = \frac{-5.84^\circ \times 100}{1 \times 23.17} = -25.19^\circ$ ,  $[M]_D^{20} = -37.78^\circ$ , were poured slowly with cooling into 50 gm. of thionyl chloride. The reaction mixture was then heated for 15 minutes on the steam bath under a return condenser. The isolation and purification were carried out exactly as in the preparation of *n*-propylphenyl chloromethane. The halide was distilled under reduced pressure (about 15 mm.), boiling at 88–89°C. The direction of rotation was the same as that of the carbinol from which it was derived.

$$[\alpha]_D^{20} = \frac{-2.81^\circ \times 100}{1 \times 9.913} = -28.34^\circ. \quad [M]_D^{20} = -47.75^\circ.$$

0.1922 gm. substance: 0.1632 gm. AgCl.

$C_{10}H_{13}Cl$ . Calculated. Cl 21.04.  
Found. " 21.00.

*Action of Hydrobromic Acid on Dextro-Isopropylphenyl Carbinol.*—5 gm. of dextro-isopropylphenyl carbinol,  $[\alpha]_D^{20} = \frac{+4.52^\circ \times 100}{1 \times 9.632} = +47.03^\circ$ ,  $[M]_D^{20} = +70.54^\circ$ , were saturated with hydrobromic acid gas at 0°C. The reaction was completed in about 15 minutes. Ice was then added and the mixture was extracted with ether. The extract was washed free from hydrobromic acid and dried over sodium sulfate. The ether was then removed and the residue fractionated under a pressure of 0.04

mm. Under this pressure the halide distilled at 65–67°C. It rotated polarized light in the same direction as the carbinol from which it was derived.

$$[\alpha]_D^{20} = \frac{+2.80^\circ \times 100}{1 \times 10.36} = +27.02^\circ. \quad [M]_D^{20} = +58.55^\circ.$$

*Action of Thionyl Chloride on Levo-n-Butylphenyl Carbinol.*—20 gm. of levo-*n*-butylphenyl carbinol,  $[\alpha]_D^{20} = \frac{-1.15^\circ \times 100}{1 \times 28.65} = -4.01^\circ$ ,  $[M]_D^{20} = -6.57^\circ$ , were poured slowly with cooling into 80 gm. of thionyl chloride. The reaction mixture was then heated for 15 minutes on the steam bath under a return condenser. The product was then isolated and purified exactly as in the case of methylphenyl chloromethane described above. It was fractionated under reduced pressure (about 15 mm.). It boiled at 121–122°C. The chloride obtained was levorotatory.

$$[\alpha]_D^{20} = \frac{-1.04^\circ \times 100}{1 \times 7.742} = -13.43^\circ. \quad [M]_D^{20} = -24.50^\circ.$$

0.1834 gm. substance: 0.1474 gm. AgCl.

$C_{11}H_{15}Cl$ . Calculated. Cl 19.43.

Found. " 19.88.

*Action of Phosphorus Pentachloride on Levo-n-Butylphenyl Carbinol.*—5 gm. of levo-*n*-butylphenyl carbinol,  $[\alpha]_D^{20} = \frac{-1.52^\circ \times 100}{1 \times 11.846} = -12.83^\circ$ ,  $[M]_D^{20} = -20.04^\circ$ , were dissolved in 10 cc. of dry chloroform. This solution was poured slowly with cooling into a suspension of 6.34 gm. of phosphorus pentachloride in 15 cc. of dry chloroform. After all the carbinol had been added, the reaction mixture was allowed to stand at room temperature for  $\frac{1}{2}$  hour. The chloroform and phosphorus oxychloride were then removed under reduced pressure at 65°C. The residue was poured into water and extracted with ether. To insure the decomposition of any unchanged phosphorus oxychloride and the removal of hydrochloric acid, the extract was washed first with dilute sodium hydroxide and then with water. It was then dried over sodium sulfate, the ether was removed, and the residue distilled under reduced pressure (about 15 mm.). It boiled at 115–116°C. The halide was levorotatory.

$$[\alpha]_D^{20} = \frac{-0.52^\circ \times 100}{1 \times 11.938} = -5.12^\circ. \quad [M]_D^{20} = -9.34^\circ.$$

# ON THE OXIDATION OF MERCAPTANS AND THIO ACIDS TO THE CORRESPONDING SULFONIC ACIDS.\*

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The object of the work on the oxidation of optically active thio compounds to the corresponding sulfo derivatives was discussed in the preceding series of articles.<sup>1</sup> It will be referred to in this place very briefly. It is desired, first, to follow the effect on optical rotation of the change in polarity of a single group attached to the asymmetric carbon atom; second, to observe in substituted carboxylic acids the difference in the rotations of the free acids and of their salts.

With regard to the first point of interest, the following observations have been made up to the present.

In the series of secondary mercaptans, all showed a change in direction of their optical rotations on oxidation to the corresponding sulfonic acids. The mercaptans thus far analyzed in this respect are, 2-mercaptobutane, 2-mercaptoisohexane, 2-mercaptohexane, and benzylphenyl mercaptomethane. From these observations it is evident that derivatives of secondary alkyls analogous in configuration, but differing markedly in the polarity of the significant groups, rotate polarized light in opposite directions. On the basis of these considerations the conclusion was drawn that secondary alcohols and secondary halides rotating in opposite directions are configurationally related.

To these data and to the conclusions previously published we may now add the data on the oxidation of methylphenyl thiome-

\* This is the eighth paper of the series on Walden inversion.

<sup>1</sup> Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lix, 473; *ibid.*, 1, 685; 1925, lxiii, 85; lxv, 515.

thane and also on the oxidation of some of its higher homologues and their isomers. Through the work of McKenzie, it is known that on halogenation of this alcohol two enantiomorphous halides can be obtained depending upon the nature of the halogenating reagent. By means of thionyl chloride a chloride was obtained which rotated in the same direction as the parent alcohol, whereas phosphorus pentachloride led to a chloride rotating in the opposite direction. Thus evidently a Walden inversion occurred in one of the two chlorides and it was not possible to ascertain in which of the two. From the preceding publication it is evident that several higher homologues of methylphenyl carbinol also behave abnormally on halogenation. Ethylphenyl carbinol behaves similarly to the lower homologue, whereas propylphenyl-, isopropylphenyl, and *n*-butylphenyl carbinols behave differently from the aliphatic secondary alcohols and from the above two alcohols, inasmuch as with either reagent they form halides rotating in the same direction as the parent alcohol. It was therefore very important to obtain data on the influence on rotation caused by the oxidation of the mercaptans of this series to the corresponding sulfonic acids. In this series an unexpected difficulty was encountered; namely, that the majority of mercaptans on oxidation were racemized. However, in the case of ethylphenyl thiomethane, the oxidation was not accompanied by complete racemization. In this case the sulfonic acid rotated in the opposite direction to that of the mercaptan.

Thus, using the same argument as that applied in connection with the series of aliphatic alcohols, it is permissible to conclude that alcohols and halides of the phenylmethyl carbinol series are configurationally related when they rotate in opposite directions.

The abnormal behavior of the alcohols of the methylphenyl carbinol series and of the methylphenyl thiomethane series is important particularly when compared with phenylbenzyl carbinol and the corresponding mercaptan. Phenylbenzyl carbinol behaves normally like an aliphatic secondary alcohol. In the methylphenyl series the mobilities of the groups attached to the asymmetric carbon atom seem much greater than in the corresponding derivatives of the aliphatic compounds, a phenomenon which may be attributed to a greater distortion of the tetrahedron of the asymmetric carbon atom.

*Monocarboxylic Acids Substituted in Position (2).*

Of this series only one acid has been observed thus far; namely, thiolactic acid. The sulfo acid prepared from it rotated in the same direction as the parent substance. However, the numerical value of the rotation of the thio acid was higher than that of the sulfo acid. Thus, in this instance, the change in polarity resulted only in a quantitative change in rotation and at first glance the observation on these two substances seemed of little value for correlating the configurations of lactic acid and of the halogen propionic acids substituted in position (2). However, on further analysis, these observations proved to possess a great significance.

It is characteristic of dextro-lactic acid, of dextro-alanine, and of other 2-hydroxy and 2-amino acids of the *l* series, that the difference between the rotations of the ionized and that of the free acid has a negative value, as seen from Table I. Thus the presence of sodium hydroxide in the solvent affects the rotations of all these substances in the same sense. Of the five substances, two (4 and 5) are definitely known to be configurationally related on the basis of direct evidence, and two (1 and 2) are regarded as configurationally related on the basis of indirect evidence. It now seems justifiable to classify dextro-bromopropionic acid in the same series with dextro-lactic acid, namely, in the *l* series.

*Substituted Dicarboxylic Acids.*

Of this class only the derivatives of succinic acid were analyzed. In Table II the rotations of the free acids and of the salts of the various derivatives are compared. In order to interpret these data it is necessary to bear in mind the progress of ionization of the individual groups in the substituted succinic acids (see Table III) with the decrease in hydrion concentration (increase in pH values).

Comparing Table III with Table II it will be noticed that in malic and aspartic acids the ionization of the carboxyls in positions (2) and (3) leads to a change in rotation towards the left. In the thio acid, a peculiarity is noted; namely, that the ionization of the carboxyl in position (2) leads to a change in rotation towards the left as in the other two instances. The ionization of the carboxyl in position (3), on the other hand, leads to a change



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in rotation to the right as compared with the rotation of the monion.

In the sulfo acid the optical change with the ionization of individual groups is the same as in the thio acid. If the ionization in the sulfo acid proceeded in the same order as in the thio acid, then the values of the fifth line of Table II would be in the following order: +57.53, +25.67, +37.80, +49.27.

Thus, the change in rotation with the ionization of the carboxyl in position (3) would be in the same sense as in the thio acid.

TABLE I.

	$\alpha$	NaOH 2 equivalents [M] <sub>D</sub> .	NaOH 1 equivalent [M] <sub>D</sub> .	Free acid [M] <sub>D</sub> .	HCl [M] <sub>D</sub> .
	<i>per cent</i>				
1. Dextro-lactic acid*.....	5		-11.88°	+1.80°	
2. Dextro-alanine†.....				+2.40°‡	+13.60°
3. Dextro-2-bromopropionic acid§.....			-9.34°	+37.96°	
4. Dextro-2-thiopropionic acid  .....		+7.32°	-5.58°	+58.98°	
5. Dextro-2-sulfopropionic acid¶.....		-3.28°	+12.84°	+13.76°	

\* Purdie, T., and Walker, J. W., *J. Chem. Soc.*, 1895, lxvii, 630.

† Clough, G. M., *J. Chem. Soc.*, 1918, cxiii, 540, and observations in this laboratory.

‡ Ionized condition.

§ Levene, P. A., and Mikeska, L. A., unpublished.

|| Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1925, lxxiii, 86.

¶ Partially racemized, unracemized [M]<sub>D</sub> = +49.50°.

That the change in rotation with the ionization of the carboxyl in position (2) proceeds normally is seen from the behavior of thio- and sulfosuccinamide (NH<sub>2</sub> group on carboxyl in position (3)). Thus, on the basis of the above considerations, the conclusion is warranted that all the above mentioned substituted succinic acids belong to the *l* series.

In addition to these older data new data are now presented on the behavior of monocarboxylic acids substituted in position (3).

TABLE II.

	c	HCl solution [M] <sub>D</sub> .	Free acid [M] <sub>D</sub> .	1 equivalent NaOH [M] <sub>D</sub> .	2 equivalent NaOH [M] <sub>D</sub> .	3 equivalent NaOH [M] <sub>D</sub> .	Amide [M] <sub>D</sub> .
	per cent						
1. Levo-malic acid*	10		-3.59°	-7.60°	-9.22°		+133.10°†
2. Dextro-bromosuccinic acid†			+142.00°‡				
3. Dextro-aminosuccinic acid§ (aspartic)		+42.60°	+7.80°	-6.65°	+14.63°		
4. Dextro-thiosuccinic acid			+73.06°	+38.04°	+48.57°	+41.09°	
5. Dextro-sulfosuccinic acid			+57.53°	+49.27°	+37.89°	+25.67°	
6. Dextro-thiosuccinamide			+89.40°	+36.25°	+28.35°		
7. Dextro-sulfosuccinamide			+46.48°	+44.60°	+2.19°		

\* Schneider, G., *Ann. Chem.*, 1881, ccvii, 266.† Walden, P., *Z. physik. Chem.*, 1895, xvii, 249.

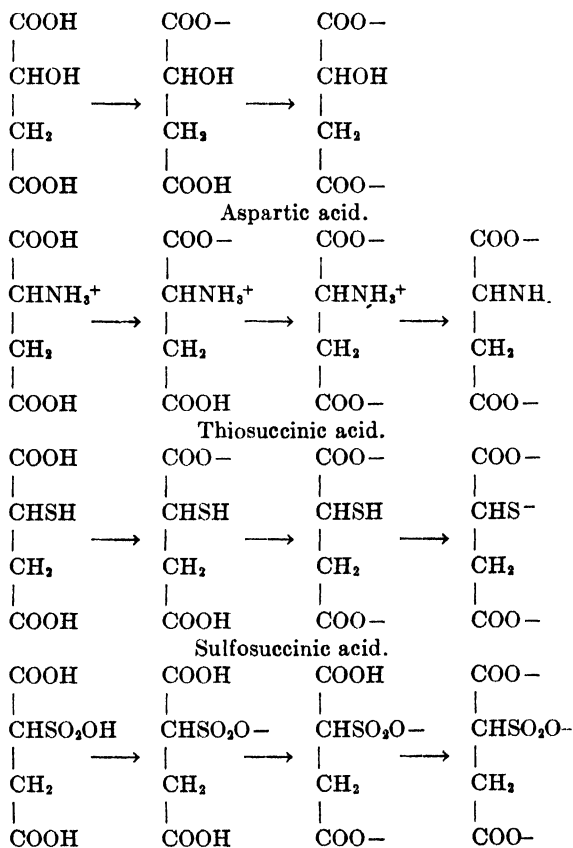
‡ In ethyl acetate.

§ Wood, J. V., *J. Chem. Soc.*, 1914, cv, 1992.|| Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1925, lxxiii, 86.

*3-Halogen Acids.*

There are fewer data on the basis of which to correlate the configuration of acids substituted in position (3). Dextro-3-hydroxybutyric acid was found configurationally related to

TABLE III.  
Malic acid.



dextro-lactic acid, and therefore was classified as an *l* acid. The rotation of this acid with the progress of ionization showed a shift to the left. Whether the same rule holds for acids substituted in position (3) by other groups cannot as yet be stated definitely. The existing data, however, indicate that in this

group of acids the direction of the change in rotation with the progress of ionization seems to depend on the polarity of the substituting group.

Thus, it was shown above that in the substituted succinic acids the change of rotation was to the left for dextro-malic and dextro-aspartic acids and to the right for dextro-thio- and dextro-sulfo-succinic acids. For the group of 3-hydroxy substituted acids, the data given in Table IV are available.

Taking it for granted that the oxidation of the 3-thio acid to the sulfonic acid proceeds without change of configuration, one may assume that on substitution of hydroxyl by halogen a similar change in optical behavior will take place. In that case, levo-

TABLE IV.

	c	HCl [M] <sub>D</sub> .	NaOH 2 equivalents [M] <sub>D</sub> .	NaOH 1 equivalent [M] <sub>D</sub> .	Free acid [M] <sub>D</sub> .
	<i>per cent</i>				
Dextro-3-hydroxybutyric acid*.....				+14.80°	+24.60°
Levo-3-aminobutyric acid†.	10	+29.70°		+14.70°	-35.00°
Levo-3-chlorobutyric " †.	10			-50.59°	-61.00°
Dextro-3-thiobutyric " ..			+23.05°	+39.33°	+49.26°
Dextro-3-sulfobutyric " ..			+19.59°	+7.04°	+6.90°

\* McKenzie, A., *J. Chem. Soc.*, 1902, lxxxi, 1402. Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxx, 49.

† Fischer, E., and Scheibler, H., *Ann. Chem.*, 1911, cccclxxxiii, 349.

3-chlorobutyric acid will be configurationally related to dextro-3-hydroxybutyric acid. From the behavior of malic and aspartic acids, on ionization of the carboxyl in position (3), it may be concluded that configurationally related 3-hydroxybutyric acid and 3-aminobutyric acid should show similar optical change with the progress of ionization of the carboxyl and hence that the dextro-hydroxy and the levo-amino acids should be configurationally related. Hence, the substances enumerated in Table IV should all belong to the *l* series.

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### SUMMARY.

1. Methylphenyl, ethylphenyl, *n*-propylphenyl, isopropylphenyl, and *n*-butylphenyl thiomethanes were prepared and oxidized to the corresponding sulfonic acids.

2. The ethylphenyl methane sulfonic acid rotates in an opposite direction to the corresponding mercaptan.

3. 3-Thiobutyric acid was resolved and the dextro-form was prepared in pure state. Its rotation was  $[M]_D = +62.26^\circ$ .

4. On oxidation, it gave dextro-3-sulfobutyric acid.

5. The latter acid was purified until its rotation reached a maximum which was found to be  $[M]_D = +6.68^\circ$ .

6. With the progress of ionization of the carboxyl the change in the rotation of the thio acid was to the left, that of the sulfo acid to the right.

7. Configurational relationships were discussed in the light of the optical behavior of the thio derivatives of the corresponding sulfonic acids in the following groups of substances: (a) in several secondary alcohols and the corresponding halides; (b) in 2-substituted propionic acids; (c) in substituted succinic acids; (d) in 3-substituted butyric acids.

8. The conclusions reached were in regard to (a) that configurationally related alcohols and halides rotate in opposite directions; in regard to (b) that dextro-lactic acid, dextro-alanine, and dextro-chloropropionic acid are configurationally related; in regard to (c) that levo-malic, dextro-chlorosuccinic, and dextro-aspartic acids are configurationally related; in regard to (d) that dextro-3-hydroxy-, levo-3-amino-, and levo-3-chlorobutyric acids are configurationally related.

9. All the acids enumerated in (8) belong to the  $l^2$  series. On the basis of other considerations, identical conclusions were reached by G. W. Clough.

10. It is realized that more experimental data are needed in order to give a firmer and more comprehensive character to the above conclusions.

<sup>2</sup> Dextro or levo refers to the direction of rotation of the undissociated acids. *d* and *l* indicate configurational relationship to *d*- and *l*-lactic acids, *d*-lactic acid being the one for which the difference between the rotation of the ionic form and that of the free acid has a positive value.

## EXPERIMENTAL.

*$\beta$ -Bromobutyric Acid.*—The  $\beta$ -bromobutyric acid used in our work was prepared according to the directions of Brulé<sup>3</sup> by passing hydrobromic acid gas through molten crotonic acid. The reaction mixture was maintained at 80–90°C. by means of a water bath, until slightly more than the calculated amount of hydrobromic acid had been absorbed. The bromo acid was then distilled under reduced pressure (about 16 mm.). At this pressure the acid distilled without decomposition at 115–116°C. as a clear colorless liquid which solidified readily on cooling to 0°C. 50 gm. of crotonic acid yielded 92 gm. of pure bromo acid.

*$\beta$ -Xanthobutyric Acid.*—200 gm. of  $\beta$ -bromobutyric acid were dissolved in 600 cc. of water, cooled thoroughly, and neutralized with 80 gm. of potassium carbonate. While the solution was still cold, 206 gm. of potassium xanthate were added. The mixture was allowed to stand overnight at room temperature. The next day 400 cc. of concentrated hydrochloric acid were added and the mixture was heated for  $\frac{1}{2}$  hour on a steam bath to decompose the unchanged xanthic acid. On cooling, the xanthobutyric acid crystallized. It was extracted with ether, washed with water, and dried over sodium sulfate. On removal of the ether the residue solidified and was used for the preparation of  $\beta$ -thiobutyric acid without any further purification. Yield = 240 gm.

*$\beta$ -Thiobutyric Acid.*—240 gm. of  $\beta$ -xanthobutyric acid were dissolved in 2.4 liters of absolute alcohol. 720 cc. of concentrated ammonia were then added and the mixture was allowed to stand at room temperature for 48 to 72 hours. The alcohol and excess of ammonia were then removed under reduced pressure, the residue was acidified with 240 cc. of concentrated hydrochloric acid, and was extracted several times with ether. The extract was washed with a little water, dried over sodium sulfate, the ether removed, and the residue distilled under reduced pressure (about 16 mm.). The thio acid distilled at 116–118°C. The substance analyzed as follows:

0.1274 gm. substance: 0.2538 gm. BaSO<sub>4</sub>.

C<sub>4</sub>H<sub>8</sub>O<sub>2</sub>S. Calculated. S 26.66.

Found. " 27.37.

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<sup>3</sup> Brulé, M., *Bull. Soc. chim.*, 1909, v, 1019.

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*Resolution of  $\beta$ -Thiobutyric Acid.*—A warm solution of 100 gm. of  $\beta$ -thiobutyric acid in 500 cc. of acetone was treated with 278.3 gm. (1 mol) of pure quinine. On cooling and stirring, the salt separated in beautifully crystalline form. The salt was then repeatedly recrystallized from acetone until further recrystallizations no longer increased the optical activity of the thio acid. To decompose the quinine salt it was suspended in water and treated with a slight excess of ammonia. The quinine was filtered off, the filtrate concentrated, and extracted several times with chloroform. The aqueous layer was then acidified and extracted with ether. The ether extract was washed with water and dried over sodium sulfate. The ether was then removed and the residue fractionated under reduced pressure (about 16 mm.). The substance distilled at 116–118°C. Its optical activity was found to be

$$[\alpha]_D^{20} = \frac{-3.26^\circ \times 100}{1 \times 7.876} = -41.05^\circ. \quad [M]_D = -49.26^\circ \text{ in } H_2O.$$

To determine the activity of the monosodium salt, 0.9858 gm. of the thio acid was treated with 8.23 cc. of  $N$  NaOH. The solution was then made up to 10 cc. This corresponds to 1.1665 gm. of monosodium salt. Hence,

$$[\alpha]_D^{20} = \frac{-3.24^\circ \times 100}{1 \times 1.1665} = -27.74^\circ. \quad [M]_D = -39.33^\circ.$$

In a similar way, the activity of the disodium salt was determined by neutralizing 0.4006 gm. of thio acid with 6.67 cc.  $N$  NaOH (2 mols) and diluting the solution to 10 cc. This corresponds to 0.5475 gm. of disodium salt. Hence,

$$[\alpha]_D^{20} = \frac{-0.77^\circ \times 100}{1 \times 5.475} = -14.06^\circ. \quad [M]_D = -23.05^\circ.$$

In another experiment a thio acid was obtained which showed the optical rotation of

$$[\alpha]_D^{20} = \frac{-3.94^\circ \times 100}{1 \times 7.592} = -51.89^\circ \text{ (in } H_2O). \quad [M]_D = -62.26^\circ.$$

The substance analyzed as follows:

0.1306 gm. substance:	0.2650 gm. $BaSO_4$ .
	$C_4H_8O_2S$ . Calculated. S 26.66.
	Found. " 26.34.

*Levo-β-Sulfobutyric Acid.*—10 gm. of levo-β-thiobutyric acid ( $[\alpha]_D^{20} = -37.81^\circ$ ) were dissolved in 150 cc. of water. 9 equivalents of barium carbonate were then added. The mixture was thoroughly cooled and treated with 7 equivalents of bromine in small amounts. The last traces of unconsumed bromine were removed by adding a drop of the thio acid. The excess of barium carbonate was filtered off and the filtrate concentrated under reduced pressure. On addition of alcohol to the hot filtrate, the barium sulfonate separated in a beautifully crystalline form. The salt was redissolved in hot water and reprecipitated with alcohol. This operation was repeated until the salt obtained was free from bromides. About three recrystallizations usually sufficed to accomplish this purification. The salt showed an optical rotation of

$$[\alpha]_D^{20} = \frac{-1.21^\circ \times 100}{1 \times 18.730} = -6.46^\circ. \quad [M]_D = -19.59^\circ.$$

To determine the rotation of the free acid and the acid salt, a given amount of the dibarium salt was treated with 2 and 1 equivalent, respectively, of hydrochloric acid. For the monosalt 2.1745 gm. of dry salt were treated with 7.17 cc. N HCl and the volume was made up to 10 cc. with water. The reading as determined in a 2 dm. tube was  $-1.01^\circ$ . This corresponds to 1.6891 gm. of monobarium salt.

$$[\alpha]_D^{20} = \frac{-1.01^\circ \times 100}{2 \times 16.891} = -2.99^\circ. \quad [M]_D = -7.04^\circ.$$

The salt analyzed as follows:

0.0937 gm. substance: 0.0683 gm. BaSO<sub>4</sub> (for Ba).

0.1547 " " : 0.1140 " " ( " S).

C<sub>4</sub>H<sub>6</sub>O<sub>3</sub>BaS. Calculated. Ba 45.28, S 10.57.

Found. " 42.89, " 10.12.

For the free acid 1.4057 gm. of the dry salt was neutralized with 9.26 cc. N HCl. This corresponds to 0.7783 gm. of free acid. Hence,

$$[\alpha]_D^{20} = \frac{-0.64^\circ \times 100}{2 \times 7.783} = -4.11^\circ. \quad [M]_D = -6.90^\circ.$$

*Dextro-β-Sulfobutyric Acid.*—Partly active dextro-β-thiobutyric acid was oxidized with bromine as described in the previous



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experiment. The barium salt was purified and the barium removed quantitatively with sulfuric acid. On concentration under reduced pressure the acid was obtained as a thick colorless syrup. This was dissolved in water and treated with 2 mols of brucine. On cooling, the brucine salt crystallized. The salt was then recrystallized from water until the activity of the acid no longer increased. The brucine salt was then decomposed with an excess of barium hydroxide. The alkaloid was filtered off and the last traces were removed by extraction with chloroform. The solution was then saturated with carbon dioxide, the barium carbonate filtered off, and the filtrate concentrated under reduced pressure. The barium salt was then isolated and purified as in the previous experiment. The salt showed an optical rotation of

$$[\alpha]_D^{20} = \frac{+0.85^\circ \times 100}{1 \times 12.982} = +6.54^\circ. \quad [M]_D = +19.84^\circ.$$

The salt analyzed as follows:

0.0944 gm. substance: 0.0690 gm. BaSO<sub>4</sub> (for Ba).

0.1419 " " : 0.1108 " " ( " S).

C<sub>6</sub>H<sub>4</sub>O<sub>6</sub>SBa. Calculated. Ba 45.28, S 10.57.

Found. " 43.01, " 10.73.

### *Data on the Rotation of Thiosuccinamide and the Corresponding Sulfo Acid.*

#### *Thiosuccinamide.*

[M] <sub>D</sub>	[M] <sub>D</sub>	[M] <sub>D</sub>
Free.	Mono-salt.	Di-salt.
+89.40°	+36.25°	+28.35°

#### *Sulfosuccinamide.*

[M] <sub>D</sub>	[M] <sub>D</sub>	[M] <sub>D</sub>
Free.	Mono-salt.	Di-salt.
+46.48°	+44.60°	+2.19°

*Optical Activity of Thiosuccinamide and Its Salts.*—0.6596 gm. of thiosuccinamide was dissolved in water and made up to a volume of 10 cc. Reading was taken in a 2 dm. tube and was found to be +7.91°. Hence,

$$[\alpha]_D^{20} = \frac{+7.91^\circ \times 100}{2 \times 6.596} = +60.00^\circ. \quad [M]_D = +89.40^\circ.$$

To determine the rotation of the mono-salt, 0.9600 gm. of the thioamide was treated with 1 equivalent of sodium hydroxide and the volume was made up to 15 cc. This corresponds to 1.1017 gm. of the monosodium salt, or to 0.7344 gm. of salt per 10 cc. Rotation determined in a 2 dm. tube was

$$[\alpha]_D^{20} = \frac{+4.64^\circ \times 100}{2 \times 7.344} = +31.59^\circ. \quad [M]_D = +36.25^\circ.$$

The activity of the disodium salt was determined as follows: 0.9212 gm. of the thioamide was treated with 2 equivalents of sodium hydroxide and the volume made up to 15 cc. This corresponds to 0.8402 gm. of the disodium salt per 10 cc. of solution. The rotation determined in a 2 dm. tube was

$$[\alpha]_D^{20} = \frac{+3.68^\circ \times 100}{2 \times 8.402} = +21.89^\circ. \quad [M]_D = +28.35^\circ.$$

*Optical Activity of Sulfosuccinamide and Its Salts.*—2.377 gm. of the neutral barium salt were dissolved in water and the volume made up to 15 cc. The reading was found to be  $+0.21^\circ$  in a 2 dm. tube. Hence,

$$[\alpha]_D^{20} = \frac{+0.21^\circ \times 100}{2 \times 15.85} = +0.66^\circ. \quad [M]_D = +2.19^\circ.$$

To determine the rotation of the acid salt (monobarium salt), the above solution was treated with 1.90 cc. of 3.76 N HCl. The total volume was 16.9 cc., and the reading in a 2 dm. tube was  $+3.76^\circ$ . This corresponds to 1.1111 gm. of the acid salt per 10 cc. Hence, for the acid salt,

$$[\alpha]_D^{20} = \frac{+3.76^\circ \times 100}{2 \times 11.111} = +16.92^\circ. \quad [M]_D = +44.60^\circ.$$

Another equivalent of normal hydrochloric acid was added. The total volume was then 18.8 cc., and the reading in a 2 dm. tube was found to be  $+3.56^\circ$ . Hence, for the free acid

$$[\alpha]_D^{20} = \frac{+3.56^\circ \times 100}{2 \times 7.482} = +23.78^\circ. \quad [M]_D = +46.48^\circ.$$

*Levo-Methylphenyl Mercaptomethane.*—A mixture of 20 gm. of dextro-methylphenyl chloromethane,  $[\alpha]_D^{20} = \frac{+0.62^\circ \times 100}{1 \times 7.363} = +8.42^\circ$ , and 3 mols of alcoholic potassium hydrogen sulfide was heated on the steam bath under a return condenser for 3 hours.

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The solution was then diluted with water and extracted with ether. The extract was washed with water and dried over sodium sulfate. When dry, the ether was removed and the residue distilled under a pressure of about 15 mm. The mercaptan boiled constantly at 95°C. The optical rotation was determined in ether solution and was found to be

$$[\alpha]_D^{20} = \frac{-0.51^\circ \times 100}{1 \times 6.272} = -8.13^\circ.$$

0.1476 gm. substance: 0.2500 gm. BaSO<sub>4</sub>.

C<sub>8</sub>H<sub>10</sub>S. Calculated. S 23.18.

Found. " 23.27.

*Methylphenyl Methane Sulfonic Acid*.—7 gm. of levo-methylphenyl mercaptomethane,  $[\alpha]_D^{20} = \frac{-0.88^\circ \times 100}{1 \times 11.018} = -7.98^\circ$ , were dissolved in 70 cc. of acetone and 10 cc. of water. Then 16.10 gm. of potassium permanganate in acetone solution were added in small amounts with cooling until the permanganate was no longer decolorized. The mixture was then heated with an occasional addition of potassium permanganate until all the oxidizing agent had been consumed. The manganese dioxide was then filtered off and the filtrate decolorized with sulfur dioxide. The solution was then evaporated to dryness under reduced pressure. The residue was first washed several times with ether, then dissolved in absolute alcohol, and finally saturated with carbon dioxide. The precipitated salt was filtered off. On addition of ether to the filtrate the potassium sulfonate separated in an amorphous form. It was recrystallized twice from water. It was found to be inactive in both neutral and acid solutions.

0.0990 gm. substance: 0.0388 gm. K<sub>2</sub>SO<sub>4</sub> (for K).

0.1107 " " : 0.1172 " BaSO<sub>4</sub> (" S).

C<sub>8</sub>H<sub>9</sub>O<sub>2</sub>SK. Calculated. S 14.30, K 17.47.

Found. " 14.54, " 17.58.

*Dextro-Ethylphenyl Mercaptomethane*.—17 gm. of levo-ethylphenyl chloromethane,  $[\alpha]_D^{20} = \frac{-6.71^\circ \times 100}{1 \times 13.327} = -50.34^\circ$ , were heated with 4 mols of alcoholic potassium hydrogen sulfide for 3 hours under a return condenser. The solution was diluted with water and extracted with ether. The extract was dried over sodium sulfate, then distilled under a pressure of 15 mm. It

boiled at 103–104°. In ether solution the mercaptan was dextro-rotatory.

$$[\alpha]_D^{20} = \frac{+2.16^\circ \times 100}{4.776 \times 1} = +45.22^\circ.$$

0.1564 gm. substance: 0.2258 gm. BaSO<sub>4</sub>.

C<sub>9</sub>H<sub>12</sub>S. Calculated. S 21.03.

Found. " 20.67.

*Levo-Ethylphenyl Methane Sulfonic Acid*.—4 gm. of dextro-ethylphenyl mercaptomethane,  $[\alpha]_D^{20} = \frac{+2.21^\circ \times 100}{1 \times 5.182} = +42.63^\circ$ ,

were dissolved in 40 cc. of acetone and 5 cc. of H<sub>2</sub>O. This was treated with 4.8 gm. of barium permanganate in acetone solution. The oxidizing agent was added in small amounts with cooling. The manganese dioxide was then filtered off and the slightly reddish solution was decolorized with an additional drop of the mercaptan. The solution was then evaporated to dryness under reduced pressure and the solid residue washed several times with ether. The residue was then recrystallized twice from water. To determine the rotation, 1.0152 gm. of substance was treated with 2 cc. of concentrated hydrochloric acid. This was then diluted to 10 cc. and the rotation was determined in a 1 dm. tube. The reading was  $-0.16^\circ$ .

$$[\alpha]_D^{20} = \frac{-0.16^\circ \times 100}{1 \times 10.152} = -1.50^\circ. \quad [M]_D = -3.00^\circ.$$

0.0985 gm. substance: 0.0856 gm. BaSO<sub>4</sub> (for S).

0.0971 " " : 0.0406 " " (" Ba).

C<sub>13</sub>H<sub>12</sub>O<sub>6</sub>Ba. Calculated. S 11.34, Ba 24.36.

Found. " 11.94, " 24.60.

*Dextro-n-Propylphenyl Mercaptomethane*.—9 gm. of *n*-propylphenyl chloromethane,  $[\alpha]_D^{20} = -40^\circ$ , were heated with 4 mols of potassium hydrogen sulfide for 3 hours under a return condenser. The mixture was diluted with water and extracted with ether. The extract was washed with water and then dried over sodium sulfate. After the ether had been removed, the residue was distilled under reduced pressure (about 15 mm.). It distilled at 132–133°C., and was dextrorotatory.

$$[\alpha]_D^{20} = \frac{+1.82^\circ \times 100}{1 \times 4.528} = +40.19^\circ.$$

0.1576 gm. substance: 0.2228 gm. BaSO<sub>4</sub>.

C<sub>10</sub>H<sub>14</sub>S. Calculated. S 19.32.

Found. " 19.42.

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*Dextro-Isopropylphenyl Mercaptomethane*.—A mixture of 10 gm. of levo-isopropylphenyl chloromethane,  $[\alpha]_D^{20} = \frac{-1.38^\circ \times 100}{1 \times 8.398} = -16.56^\circ$ , and 3 mols of alcoholic potassium hydrogen sulfide was heated for 4 hours under a return condenser. Water was then added and the mixture extracted with ether. The extract was washed with water and dried over sodium sulfate. When dry, the ether was removed and the residue distilled under a pressure of about 15 mm. All the substance distilled at 92–96°C. The mercaptan was dextrorotatory.

$$[\alpha]_D^{20} = \frac{+1.02^\circ \times 100}{1 \times 6.728} = +15.16^\circ \text{ (in ether).}$$

0.1399 gm. substance: 0.1800 gm. BaSO<sub>4</sub>.

C<sub>10</sub>H<sub>13</sub>S. Calculated. S 19.32.

Found. " 17.76.

*Dextro-n-Butylphenyl Mercaptomethane*.—15 gm. of levo-*n*-propylphenyl chloromethane,  $[\alpha]_D^{20} = \frac{-2.92^\circ \times 100}{1 \times 16.752} = -17.43^\circ$ , were heated with 3 mols of alcoholic potassium hydrogen sulfide for 3 hours under a return condenser. The mercaptan was isolated as described in the experiment above. It distilled at 122–125°C. under a pressure of about 15 mm. It was dextrorotatory in ether solution.

$$[\alpha]_D^{20} = \frac{+1.95^\circ \times 100}{1 \times 10.870} = +17.94^\circ.$$

0.1560 gm. substance: 0.1900 gm. BaSO<sub>4</sub>.

C<sub>11</sub>H<sub>16</sub>S. Calculated. S 17.77.

Found. " 16.70.

*Oxidation of n-Propylphenyl Mercaptomethane and Some of Its Homologues*.—The oxidation of optically active *n*-propyl-, isopropyl-, and *n*-butylphenyl mercaptomethanes was carried out with potassium and barium permanganates as described in the oxidation of ethylphenyl mercaptomethane. In every case, however, the sulfonic acid obtained was inactive. Oxidation was also carried out with potassium dichromate and with hydrogen peroxide. But with these, as with the permanganates, the oxidation products obtained were inactive.

# THE LABILITY OF THE SULFUR IN CYSTINE DERIVATIVES AND ITS POSSIBLE BEARING ON THE CONSTITUTION OF INSULIN.

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## I.

Bergmann and his coworkers have published a series of investigations<sup>1</sup> on "molecular rearrangements of peptide-like bodies," which seem destined to influence our views on the structure of the protein molecule. In the course of these studies, Bergmann and Stather<sup>2</sup> recently synthesized the dianhydride of dialanyl-L-cystine (IV).

This new dioxopiperazine is characterized by the following remarkable behavior: when treated with dilute NaOH at room temperature, its sulfur is split off together with hydrogen, and a crystalline, sulfur-free reaction product results. Bergmann was able to show that this product is identical with a compound which he had previously obtained from alanyl-serine, namely 3-methylene-6-methyl-2,5-dioxopiperazine ( $C_6H_8O_2N_2$ )<sub>x</sub> (V).

Among the known cystine (I) derivatives and the compounds closely related to cystine, isocystine<sup>3</sup> (II) is the only one which contains sulfur in such a labile form. In fact the sulfur in the latter seems to be even more loosely bound than in dialanyl-cystine dianhydride, for isocystine will give off sulfur on boiling with dilute *acid*  $CuSO_4$  solution.<sup>3</sup>

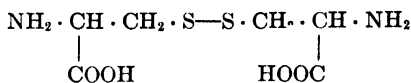
The remarkable difference in the lability of the sulfur in cystine and its peptides on the one hand, and in dialanyl-cystine dianhydride on the other, is due, Bergmann believes, to the insertion of the cystine molecule into the dioxopiperazine ring. Whether, however, a definite difference exists between cystine and its ordinary peptides in respect to the combination of their sulfur, does not

<sup>1</sup> First communication, Bergmann, M., and Brand, E., *Ber. chem. Ges.*, 1926, lvi, 1280.

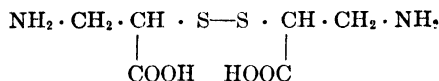
<sup>2</sup> Bergmann, M., and Stather, F., *Z. physiol. Chem.*, 1926, clii, 189.

<sup>3</sup> Gabriel, S., *Ber. chem. Ges.*, 1905, xxxviii, 630.

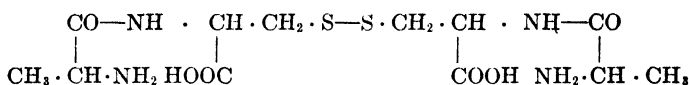
seem to have been investigated. Emil Fischer<sup>4</sup> established only that peptides of cystine give a positive lead sulfide test. Bergmann<sup>5</sup> also, in his recent publication, does not go beyond the general statement that free cystine appears to split off its sulfur more slowly than the proteins. A comparative study of cystine and, *e.g.*, dialanyl-cystine (III) would show in all probability only a moderate difference in their sulfur lability. But it is just such slight differences that are quite frequently of considerable biological importance. Thus Abel<sup>5-7</sup> and his coworkers were guided in their successful work on the purification and crystallization of insulin by their finding that the sulfur in the insulin molecule is slightly, but definitely, more labile than cystine sulfur.



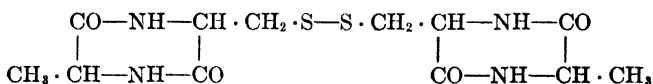
I. Cystine.



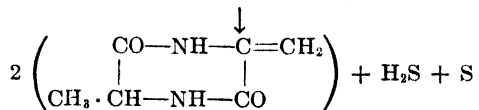
II. Isocystine.



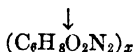
III. Dialanyl-cystine.



IV. Dialanyl-cystine dianhydride.



V. 3-Methylene-6-methyl-2,5-dioxopiperazine.



<sup>4</sup> Fischer, E., and Gerngross, O., *Ber. chem. Ges.*, 1909, xlii, 1488.

<sup>5</sup> Abel, J. J., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, 1925, xxv, 423.

<sup>6</sup> Abel, J. J., Geiling, E. M. K., Alles, G., and Raymond, A., *Science*, 1925, lxvii, 169.

<sup>7</sup> Abel, J. J., *Proc. Nat. Acad. Sc.*, 1926, xii, 132.

A distinct difference between cystine and dialanyl-cystine is immediately evident if these substances are left standing for some time at room temperature with *N* alkali-lead acetate mixture. Bergmann<sup>2</sup> states that under such conditions a dialanyl-cystine

TABLE I.  
*Lability of the Sulfur in Cystine Derivatives and in Insulin.*

Substance.	Total S split off under different conditions.		
	Boiling with copper sulfate in 2 <i>N</i> HCl for 20 minutes.	Sodium carbonate sulfur according to Abel and Geiling <sup>5</sup>	* Standing with <i>N</i> NaOH-Pb acetate at room temperature.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Cystine (I).....	None.	2.5* 2.8	None (after 24 hrs. at 24°C.).
Isocystine (II).....	++†	++†	++†
Dialanyl-cystine (III).	None.	18.6	4.7‡ (after 7 hrs. at 18°C.). 14.3 (after 24 hrs. at 24°C.).
Dialanyl-cystine dianhydride (IV).....	"	91.8	71‡ (after 30 min. at 18°C.).
ML.§.....	"	61.5	23.3 (after 30 min. at 24°C.).
Insulin. ¶.....	"	35.6 38-47*	9.3 (after 7 hrs. at 24°C.).

\* Abel.<sup>5</sup>

† Gabriel.<sup>3</sup>

‡ Bergmann.<sup>2</sup>

§ See p. 385.

¶ See p. 386.

solution takes on a brown color within 30 minutes, and after several hours precipitates definite amounts of PbS. A cystine solution, however, according to our observation, remains colorless under identical conditions for more than 24 hours and does not precipitate any PbS. When dialanyl-cystine is boiled for some



time with 0.1 N sodium carbonate, a definite yellow color develops, and addition of dilute acids now liberates hydrogen sulfide. Abel and Geiling<sup>5</sup> designate the sulfur that is split off by such treatment as "sodium carbonate sulfur." These authors find that under the conditions of their method cystine will give off only 2.5 per cent of its sulfur as sodium carbonate sulfur. Under identical conditions, dialanyl-cystine, however, splits off 18.5 per cent of sodium carbonate sulfur, almost eight times<sup>8</sup> as much (see Table I). No observations have been recorded, as yet, concerning the nature of the product that is obtained after the removal of the sulfur from dialanyl-cystine.

It is evident, therefore, that the introduction of two alanyl groups into the amino groups of cystine is accompanied by a distinct increase in the lability of the sulfur. The order of magnitude of this increase in other N substituted peptides of cystine will depend on the size and character of the amino acids introduced. A systematic study of the lability of the sulfur in such substituted compounds seems desirable, particularly that of the physiologically important diglutaminyl-cystine (glutaminyl-cysteine = glutathione); for it may be imagined that at certain H ion concentrations the splitting off of hydrogen sulfide may constitute a complicating factor in the behavior of glutathione and similar compounds.

Moreover, it seems important to study the influence which substitution in the COOH groups of cystine and its N peptides will exert on the lability of the sulfur. The study of such compounds, *e.g.*, tetra-alanyl-cystine, should yield additional information regarding the causes of the extreme lability of the sulfur in dialanyl-cystine dianhydride. We may then be able to determine to what extent the behavior of the latter compound is due to the presence of the dioxopiperazine ring as such.

The available evidence shows that in certain cystine derivatives the sulfur is much more loosely bound than in cystine itself. The amount of sulfur that is split off under identical conditions from cystine or a cystine derivative, while definite, varies to a great extent according to the compound. If, therefore, we find that the sulfur lability of a protein, polypeptide, or polypeptide-like compound of unknown structure differs from that of cystine,

<sup>8</sup>This calculation is only approximately accurate, since corrections should be made on account of the different amounts of COOH groups per gm. of cystine and of dialanyl-cystine.

this finding cannot be interpreted as evidence that (1) a sulfur-containing amino acid other than cystine is present, or that (2) the sulfur in such compounds is present in more than one form of combination.

#### EXPERIMENTAL.

Dialanyl-*l*-cystine is prepared according to Fischer and Suzuki,<sup>9</sup> dialanyl-*l*-cystine dianhydride as described by Bergmann and Stather.<sup>2</sup> The last stage in the preparation of the latter compound consists in saturating a methyl alcoholic solution of dialanyl-cystine-dimethyl ester chlorohydrate with dry ammonia. From this solution the dialanyl-cystine dianhydride crystallizes, and a methyl alcoholic mother liquor is left. From this mother liquor dry ether (700 cc.) precipitates 3.2 gm. of a slightly yellowish substance, which will be designated as ML. We did not attempt to purify this substance further, which still contains about 20 per cent of ammonium chloride. ML is easily soluble in water and alcohol, a very slight turbidity may be removed by centrifuging. ML gives a strong biuret reaction and positive reactions with picric acid and *m*-dinitrobenzene, but only a slight reaction with *m*-dinitrobenzoic acid, and a negative nitroprusside reaction; it contains 11.4 per cent of sulfur; 0.212 gm. of ML yields 0.1759 gm. of BaSO<sub>4</sub>. The sulfur in ML is very loosely bound; if 100 mg. of ML are treated at 20°C. with 15 cc. of *N* NaOH containing 0.25 gm. of lead acetate, a precipitate of PbS is immediately formed that is filtered off after 30 minutes, and which then amounts to 0.0198 gm. of PbS = 23.3 per cent of the total sulfur. Estimation of sodium carbonate sulfur according to Abel and Geiling<sup>5</sup> shows that by such treatment 61.5 per cent of the total sulfur is split off; 50 mg. of ML boiled for 45 minutes with 10 cc. of 0.1 *N* sodium carbonate solution split off 0.0035 gm. of sulfur. According to its preparation and reactions ML is a derivative of dialanyl-cystine and probably contains no dioxopiperazine ring;<sup>10</sup> nevertheless the lability of its sulfur is almost as high as that of the sulfur in dialanyl-cystine dianhydride. The solubility in water of dialanyl-cystine dianhydride is not increased by the presence of ammonium chloride.

*Cystine*.—Estimation of sodium carbonate sulfur:<sup>5</sup> 50 mg. boiled for 45 minutes with 10 cc. of 0.1 *N* sodium carbonate solution split off 0.37 mg. of S = 2.8 per cent of the total S. 50 mg. boiled for 45 minutes with 10 cc. of 0.125 *N* sodium carbonate solution split off 0.65 mg. of S = 4.9 per cent of the total S. 50 mg. boiled for 45 minutes with 10 cc. of 0.15 *N* sodium carbonate solution split off 1.26 mg. of S = 9.4 per cent of the total S.

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<sup>9</sup> Fischer, E., and Suzuki, U., *Ber. chem. Ges.*, 1904, xxxvii, 4575.

<sup>10</sup> Bergmann, M., and Stather, F., (*Ann. Chem.*, 1926, cdxlviii, 32) recently synthesized the dianhydride of dileucyl-*l*-cystine. It seems that, notwithstanding the presence of the dioxopiperazine ring, the sulfur in dileucyl-cystine dianhydride is much more stable than in the corresponding alanyl anhydride and in substance ML.

100 mg. dissolved in 30 cc. of *N* NaOH containing 0.4 gm. of lead acetate and kept for 24 hours at 24°C. do not precipitate any PbS.

*Dialanyl-Cystine*.—Estimation of sodium carbonate sulfur: 50 mg. boiled for 45 minutes with 10 cc. of 0.1 *N* sodium carbonate solution split off 1.56 mg. of S = 18.6 per cent of the total S.

100 mg. dissolved in 20 cc. of *N* NaOH containing 0.3 gm. of lead acetate and kept for 24 hours at 24°C. precipitate 0.0179 gm. of PbS = 14.3 per cent of the total S.

*Dialanyl-Cystine Dianhydride*.—Estimation of sodium carbonate sulfur: 50 mg. boiled for 45 minutes with 10 cc. of 0.1 *N* sodium carbonate solution split off 8.5 mg. of S = 91.8 per cent of the total S.

*Insulin*.—The insulin used in our experiments was Iletin Lilly, Lot 769999, containing 440 units per cc. and 21 units per mg. of solids. The preparation was precipitated three times at the isoelectric point with the help of  $\frac{1}{2}$  *N* pyridine and then dried *in vacuo*. The content of total sulfur in our preparation was 2.7 per cent; 0.0978 gm. yields 0.0192 gm. of BaSO<sub>4</sub>.

Estimation of sodium carbonate sulfur: 50 mg. boiled for 45 minutes with 10 cc. of 0.1 *N* sodium carbonate solution split off 0.48 mg. of S = 35.6 per cent of the total S.

100 mg. dissolved in 20 cc. of *N* NaOH containing 0.3 gm. of lead acetate and kept for 7 hours at 24°C. precipitate 0.0019 gm. of PbS = 9.3 per cent of the total S.

## II.

The previous discussion attains a special significance when correlated with certain facts now established as to the probable constitution of insulin. That sulfur and cystine are present in insulin preparations of varying degrees of purity has been observed by a number of investigators, but only Abel and Geiling<sup>5</sup> succeeded in establishing a definite relation of the sulfur reaction<sup>11</sup> to the actual active principle. These authors found that their highly purified, active preparations of insulin and their crystalline insulin are characterized by the presence in them of sulfur in a remarkably labile form of combination. They "have furnished proof that when an 'insulin' of relatively high unitage is boiled for a short time with *N*/10 Na<sub>2</sub>CO<sub>3</sub> the resulting physiological inactivation is always associated with an alteration in the linkage of a part if not all of the sulphur that properly belongs to the hormone. After such treatment with sodium carbonate a new property appears in the altered insulin in that it shows extraordinary sensitivity to very dilute acids which now liberate hydrogen

<sup>11</sup> Cf. Taylor, T. C., Braun, C. E., and Scott, E. L., *Am. J. Physiol.*, 1925, lxxiv, 539.

sulphide from it. Previous to this alkaline treatment these same acids applied in greater concentration and with heat fail to evolve hydrogen sulphide from insulin." This behavior of insulin induces Abel and Geiling<sup>5</sup> to point out that insulin presents some analogies to the thiopiperazine and thiopolypeptide derivatives that have been synthesized by Johnson and Burnham.<sup>12</sup> In regard to the remarkable lability of the sulfur in insulin the question suggests itself to Abel and Geiling<sup>5</sup> "to what extent, if any, the islets of Langerhans are dependent upon the presence in our food of a special labile sulphur compound, a precursor indispensable for the elaboration of the hormone, in the absence of an adequate supply of which, pathological alterations in the cells of the islets of Langerhans would take place."

In the light of the previous investigations of the different behavior of cystine, dialanyl-cystine, and dialanyl-cystine dianhydride, Abel's observations on the lability of the sulfur in insulin do not offer conclusive evidence that the sulfur-containing building stone of the insulin molecule is different from cystine, and that the sulfur in the insulin molecule is present in more than one form of combination. While the possibility of cystine being a component of the insulin molecule gains in probability, we have not been able to further materially the decision of this question, partly<sup>13</sup> for lack of material.

In the acid hydrolysis of 1 gm. of insulin (approximately 21,000 units) 150 mg. of dark brown crystals<sup>14</sup> were obtained on neutralization, which gave a positive Millon reaction and a very strong reaction for lead-blackening sulfur. The further purification of this material, however, was difficult. From about one-half of the material only 18 mg. of slightly yellowish crystals were obtained, which looked microscopically like cystine platelets and showed a rotation of  $[\alpha]_D^{25} = -212.5^\circ$ .

<sup>12</sup> Johnson, T. B., and Burnham, G., *J. Biol. Chem.*, 1911, ix, 449.

<sup>13</sup> Cf. Hopkins on glutathione (Hopkins, F. G., *Biochem. J.*, 1921, xv, 286 (p. 294)): "As all who have experience of the matter are aware, the quantitative separation of cystine from other amino-bodies is always difficult."

<sup>14</sup> Needles in bunches, possibly similar to the tyrosine-containing cystine needles, obtained from cystine stones. Cf. Neuberg, C., and Mayer, P., *Z. physiol. Chem.*, 1905, xlv, 472, and Fischer, E., and Suzuki, U., *Z. physiol. Chem.*, 1905, xlv, 405.

As to the lability of the sulfur in insulin, Table I shows that insulin bears more resemblance to dialanyl-cystine than to dialanyl-cystine dianhydride. It is true that insulin splits off somewhat more sulfur than dialanyl-cystine does, but treatment with an alkali-lead acetate mixture at room temperature indicates that the sulfur in insulin is by no means as labile as it is in dialanyl-cystine dianhydride. As has been pointed out before, our present knowledge of the manner in which sulfur lability in cystine derivatives depends on their constitution is very limited. It is, therefore, not possible to decide by a comparative study of the sulfur lability alone whether or not the dioxopiperazine ring is present in our insulin preparation.

The supposition, however, that the high lability of the sulfur in insulin may be due to the presence of the dioxopiperazine ring in it seems to be strengthened by Abderhalden's<sup>15</sup> finding that insulin gives a positive picric acid reaction. Our insulin preparation (approximately 21 units per mg., probably much purer than that which Abderhalden used) gives not only a strongly positive picric acid reaction, but also a positive test with *m*-dinitrobenzene, and a slight reaction with *m*-dinitrobenzoic acid. In order to evaluate the significance of these color reactions in respect to insulin, it was necessary for us to concern ourselves more thoroughly with them.

In a series of investigations on the anhydride structure of proteins, Abderhalden and Komm<sup>16,17</sup> have recently studied the reaction with *m*-dinitrobenzene compounds and the picric acid reaction<sup>18</sup> of proteins, polypeptides, amino acids, and dioxopiperazines. In these tests nearly all proteins and dioxopiperazines give a positive reaction, while polypeptides and amino acids are negative, excepting cystine and its peptides, which give a positive color reaction with dinitrobenzene compounds. Abderhalden is inclined to interpret the results of these color reactions as evidence that the dioxopiperazine ring is present in proteins. He realizes, however, that these color reactions are extremely sensitive, so that nothing can be stated as yet regarding the amount in which the color-forming groups are present in the protein molecule. We should

<sup>15</sup> Abderhalden, E., *Z. physiol. Chem.*, 1924, cxli, 304.

<sup>16</sup> Abderhalden, E., and Komm, E., *Z. physiol. Chem.*, 1924, cxxxix, 181.

<sup>17</sup> Abderhalden, E., and Komm, E., *Z. physiol. Chem.*, 1924, cxl, 99.

<sup>18</sup> Cf. Sasaki, T., *Biochem. Z.*, 1921, cxiv, 63.

like to mention that, contrary to Abderhalden,<sup>16,17</sup> arginine, tryptophane, histidine, and glutamic acid give positive results with one or the other of the reagents, when tested as described by Abderhalden (see Table II).

The fact that certain compounds, particularly such as contain sulfur, develop a yellow or brown color on boiling with sodium carbonate solution, which color sometimes masks a positive reaction, induced us to modify slightly the technique of the tests. In the tests, we use picric acid and *m*-dinitrobenzene in saturated solution, while *m*-dinitrobenzoic acid (1.3.5) is used in a solution containing 1 gm. in 100 cc. of 0.05 N NaOH: The substance to be tested (approximately 10 mg.) is boiled with 2 to 3 cc. of saturated sodium carbonate solution for about 1 minute and is immediately layered carefully with 0.5 to 1 cc. of a solution of the reagent. If the reaction is positive, a distinct ring forms at the place of contact, which in case of a strongly positive reaction may spread throughout the whole upper layer within a short time. A positive picric acid reaction yields a dark orange to red ring, with the dinitro compounds a purple to reddish brown one.

Table II shows that in our modification the color reactions yield results quite different from those obtained by Abderhalden. Only substances like creatinine, glucose, pyruvic acid, and dioxopiperazines give a positive reaction, while all the tested proteins and amino acids, excluding cystine, are negative. The attempt, therefore, to use these color reactions in establishing evidence for the existence of dioxopiperazines in proteins—as far as tested—must be considered a failure. But it seems doubtful, whether the negative results of the color tests in themselves justify the conclusion that no dioxopiperazine rings are present in the tested proteins. However, boiling of proteins with saturated sodium carbonate solution for a short time may possibly cause the formation of a small amount of dioxopiperazines or other CO compounds.

As regards the color reactions of sulfur-containing substances, Table II shows that dithiodiglycolic acid, cystine, dialanyl-cystine, substance ML, and insulin give relatively strong reactions with picric acid and *m*-dinitrobenzene, but only a slight one with *m*-dinitrobenzoic acid. The sulfur-containing dioxopiperazine (dialanyl-cystine dianhydride), however, reacts strongly also with *m*-dinitrobenzoic acid. The positive reactions of the former

TABLE II.  
*Color Reactions.*

Substance.	Picric acid.		<i>m</i> -Dinitrobenzene.		<i>m</i> -Dinitrobenzoic acid (1.3.5).	
	Ring test.	Boiling.	Ring test.	Boiling.	Ring test.	Boiling.
Glucose.....	++	++	++	++	+	+
Creatinine.....	++	++	++	++	+	+
Pyruvic acid.....	++	++	+	+	++	++
Dithiodiglycolic acid.....	++	++	++	Orange-brown.	+	Orange-brown.
Glycocol..	-	-	-	-	-	-
“ ester hydrochloride.....	-	-	-	-	-	-
Leucine.....	-	-	-	-	-	-
Arginine.....	-	+	-	+	-	-
Glutamic acid.....	-	-	-	+	-	-
Aspartic “.....	-	-	-	-	-	-
Tyrosine.....	-	-	-	-	-	-
Tryptophane.....	-	+	-	+	-	-
Histidine.....	-	+	-	-	-	-
Cystine.....	-	+	+	+	+	Orange.
Glycine anhydride.....	++	++	++	++	++	++
Dialanyl-cystine dianhydride.....	++	++	++	Orange.	++	Orange-brown.
ML.†.....	++	++	++	“	+	Orange.
Dialanyl-cystine.....	+	++	++	Orange-brown.	+	Orange-brown.
Insulin§.....	++	++	++	“	+	“

Serum albumin.....	-	+	-	+	-	-	Yellow +?
Egg albumin.....	-	+	-	+	-	-	-
Casein.....	-	+	-	+	-	-	+
Gelatin.....	-	+	-	+	-	-	-
Edestin.....	-	+	-	+	-	-	-
Zein.....	-	-	-	-	-	-	-
Gliadin.....	-	+	-	+	-	-	-

\* Reaction very weak, on boiling orange.

† In dilute solution negative (orange-brown).

‡ See p. 385.

§ See p. 396.



compounds are possibly due to the formation of pyruvic acid (for reactions see Table II).

On the other hand, the same behavior as shown by insulin—positive with picric acid and *m*-dinitrobenzene, slightly positive or negative with *m*-dinitrobenzoic acid—has been established by Abderhalden<sup>17</sup> for N-substituted dioxopiperazines. Although the possibility that the insulin molecule may contain a dioxopiperazine ring or a carbonyl group has to be kept in mind, still it would appear that the presence of labile sulfur is sufficient to account for the color reactions obtained.

As regards the manner in which the sulfur is removed from the insulin molecule by alkali, a lead as to the explanation of this phenomenon may be found in Bergmann's work on the sulfur-containing dioxopiperazine. If the insulin molecule is broken up by dilute alkali in a similar way as dialanyl-cystine dianhydride (IV, V), it is obvious that the physiological activity must be irreparably destroyed in such a complete rupture. It should be noted, however, that by treatment with *N* alkali at room temperature only relatively small amounts of sulfur are split off from insulin (see Table I). It is therefore possible that other factors besides the removal of the sulfur play a part in the alkaline destruction of the physiological activity of insulin.

Abel and Geiling's<sup>5</sup> finding of the high lability of the sulfur in insulin led them to suspect that the presence of a special labile sulfur compound in the food might be necessary for the proper functioning of the islands of Langerhans; *i.e.*, for the production of insulin.<sup>19</sup> Considering the previous discussion of the lability of the sulfur in cystine derivatives, and the consequent possibility of insulin being a cystine derivative, the lability of insulin sulfur does not afford sufficient evidence upon which to base such a hypothesis. Moreover, it seems not yet certain that the (labile) sulfur group, in spite of its analytical and chemical importance, is the carrier of the physiological activity within the insulin molecule. In this connection it is interesting to note that neither dialanyl-cystine nor dialanyl-cystine dianhydride has any influence on the blood sugar level of a fasted rabbit when those substances are injected intravenously<sup>20</sup> (see Table III).

<sup>19</sup> Cf. Bürgi, E., and Gordonoff, T., *Klin. Woch.*, 1926, v, 466.

<sup>20</sup> We are indebted to Dr. M. M. Harris for his help in these experiments.

These negative results, however, are not unequivocal. The above mentioned substances might fail to act on the blood sugar level even though it had already been established that the sulfur group was the physiologically active group of insulin, and though it had been proven that the constitution of these substances was similar to that of insulin.

For it is still undecided whether insulin is directly involved in the enzymatic processes of carbohydrate metabolism or if it is concerned only in their nervous control. Granted the first possi-

TABLE III.

*Effect of Dialanyl-Cystine and of Dialanyl-Cystine Dianhydride on the Blood Sugar Level of Fasted Rabbits.*

Time after injection.	Blood sugar.*				
	Dialanyl-cystine.			Dialanyl-cystine dianhydride.	
	Rabbit 1. Weight 2900 gm. 50 mg. intravenously.	Rabbit 2. Weight 3200 gm. 125 mg. intra- venously.	Rabbit 3. Weight 1200 gm. 100 mg. intra- venously.	Rabbit 4. Weight 1440 gm. 20 mg. intra- venously.	Rabbit 5. Weight 1130 gm. 20 mg. intra- venously + 50 mg. intra- peritoneally.
hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
-1	143	158	134	131	123
0	158	149	128	130	127
1	180 (Struggled.)	137	128	144	128
2	139	147	121	124	127
4	139	137	123	115	128
24	141	127	136	129	132

\* Folin's method.

bility to be true, the highly specific structure of enzymes and of substances participating in enzymatic processes renders it improbable that a compound, even closely related to insulin, would show an insulin-like action. Substances, however, which differ considerably in their constitution (*e.g.* pyrocatechol, epinephrine) may be similar in their effects on the visceral nervous system. Therefore, if insulin should act on the nervous system, then substances similar in constitution to insulin are more likely to produce a lowering of the blood sugar level which can be counteracted by glucose.

## SUMMARY.

1. Comparative experiments on the lability of the sulfur in cystine, dialanyl-cystine, dialanyl-cystine dianhydride, and insulin were carried out (a) by estimation of the per cent of the total S split off by treatment with N alkali-lead acetate mixture at room temperature (Bergmann), and (b) by estimation of the sodium carbonate sulfur according to Abel and Geiling.

2. The amount of sulfur that is split off under identical conditions from cystine or a cystine derivative, while definite, varies to a great extent according to the compound.

3. The finding that a protein, polypeptide, or polypeptide-like compound of unknown structure shows a sulfur lability different from that of cystine cannot be interpreted as evidence that (a) a sulfur-containing amino acid other than cystine is present, or that (b) the sulfur in such compounds is present in more than one form of combination. Abel's findings on the high degree of lability of insulin-sulfur are not opposed to cystine being a constituent of the insulin molecule.

4. At certain H ion concentrations the splitting off of hydrogen sulfide may constitute a complicating factor in the behavior of glutathione and similar compounds.

5. A modification in the technique of the color reactions for carbonyl groups with picric acid, *m*-dinitrobenzene, and *m*-dinitrobenzoic acid (1.3.5) is proposed (ring test).

6. In this modification ordinary CO compounds, dioxopiperazines, and certain sulfur compounds give positive reactions, while all the tested *proteins* and amino acids are negative, with the exception of cystine.

7. It is suggested that the positive reactions of sulfur compounds are possibly due to the formation of pyruvic acid.

8. The insulin preparation used (21 units per mg.) gives a positive picric acid reaction (Abderhalden), a positive reaction with *m*-dinitrobenzene, and a moderately positive reaction with *m*-dinitrobenzoic acid. Although the possibility that the insulin molecule may contain a carbonyl group or a dioxopiperazine ring has to be kept in mind, it appears that the presence of labile sulfur is sufficient to account for the color reactions obtained.

9. It cannot yet be decided whether or not the labile sulfur

group is the carrier of the physiological activity within the insulin molecule.

10. It is possible that other factors besides the removal of the sulfur play a part in the alkaline destruction of the physiological activity of insulin.

11. Dialanyl-cystine and dialanyl-cystine dianhydride injected intravenously have no influence on the blood sugar level of fasted rabbits.

*Note.*—While this paper was in press, Campbell and Geiling's publication on "Labile Sulphur in the Blood"<sup>21</sup> appeared.

These authors state that attempts to isolate the labile sulfur-containing moiety of the blood by the employment of the ordinary protein-precipitating agents proved unsuccessful. They were able to account for the total labile sulfur of the serum in the combined labile sulfur content of its protein constituents. Campbell and Geiling's failure to obtain in the protein-free filtrate any body containing sulfur in a labile form is probably due to their employing too small amounts of blood in their determinations. In view of these results, it was thought advisable to include in this paper some preliminary data on the lability of the sulfur in glutathione, although our oxidized glutathione, which we prepared from defibrinated ox blood, still contained impurities, its sulfur-content being 8.6 per cent instead of the theoretical figure of 12.87 per cent.

*Oxidized Glutathione.*—Estimation of sodium carbonate sulfur: 50 mg. boiled for 45 minutes with 10 cc. of 0.1 N sodium carbonate solution split off 1.34 mg. of S = 31.2 per cent of the total S. 100 mg. dissolved in 20 cc. of N NaOH containing 0.3 gm. of lead acetate and kept for 3 hours at 20°C. precipitate 0.0087 gm. of PbS = 13.6 per cent of the total S.

In spite of the impurity of our material, we believe it is safe to state that the sulfur in oxidized glutathione is far more labile than in cystine. It seems possible that the sulfur in glutathione is even more loosely bound than it is in dialanyl-cystine and in insulin (see Table I).

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<sup>21</sup> Campbell, D., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, 1926, xxviii, 389.



## ON THE ORIGIN OF GLYCURONIC ACID IN THE ORGANISM.

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The origin of glycuronic acid in the normal organism is not known although this problem has engaged the attention of various investigators for the past 50 years. The close chemical relationship of glycuronic acid to glucose makes it appear probable that it is derived from a carbohydrate precursor. With this idea in mind the synthesis of conjugated glycuronic acid was studied in depancreatized dogs (1). It was found that the production of glycuronic acid in the diabetic animal was accompanied by a corresponding decrease in urinary glucose, indicating that glycuronic acid was produced from that portion of the protein molecule which would otherwise have gone to glucose. In the diabetic animal, therefore, glucose and glycuronic acid have a common precursor. It still remains to be determined, however, whether a normal organism can produce glycuronic acid from carbohydrate, or whether it must, like the diabetic organism, synthesize it from protein. The solution of this problem is comparatively simple, for if glycuronic acid is derived from carbohydrate its synthesis should be accomplished without any marked augmentation of nitrogen metabolism. In other words the excretion of urinary nitrogen should not be increased after the ingestion of a glycuronogenic drug provided the organism is furnished with sufficient carbohydrate.

In carrying out this study, borneol was used in a few experiments, but benzoic acid was the chief substance employed since it can induce in the dog the production of relatively large quantities of glycuronic acid without producing any perceptible untoward effects. In using this drug, however, it is necessary to consider the

effects that benzoic acid has on metabolism, as well as the relationship between the synthesis of hippuric acid and nitrogen catabolism. Unfortunately, in much of the research which has been done on these problems, the production of glycuronic acid monobenzoate was either entirely overlooked or greatly underestimated. In fact, it was a common practice to record total combined benzoic acid as hippuric acid. How erroneous such an assumption may be, can readily be comprehended when one considers that the dog can easily conjugate over 70 per cent of moderately large doses of benzoic acid with glycuronic acid (2). Also in other animals glycuronic acid monobenzoate may constitute a considerable portion of the combined benzoic acid. Csonka (3) found that in the pig as much as 45 per cent of the benzoic acid was conjugated with glycuronic acid, and very recently Griffith (4) reported that also in the rabbit one-third of the benzoic acid may be thus combined. Neuberg (5) has found that even in man there is a considerable discrepancy between the total combined benzoic acid and hippuric acid.

In considering the effects of benzoic acid on nitrogen metabolism, it must be remembered that the species of animal employed constitutes a factor of utmost importance. The rabbit has been used more perhaps than any other animal, and it represents the type of organism in which the faculty to synthesize hippuric acid is very well developed. The work of Salkowski (6), Cohn (7), Pribram (8), Wiechowski (9), Ringer (10), as well as others shows that in the rabbit benzoic acid does cause an increased nitrogen excretion. It is difficult to determine how much of the augmented nitrogen metabolism can be attributed to the direct stimulation of benzoic acid on nitrogen metabolism and how much to the increased protein catabolism for the purpose of furnishing sufficient glycine for the production of hippuric acid.

The studies carried out on other animals, especially dogs, have failed to yield concordant results. Among the various factors that may account for these varied findings, diet is perhaps the most important. This was early recognized by Salkowski (11) who remarked that the state of nutrition was a factor which influenced the action of benzoic acid on protein catabolism. McCollum and Hoagland (12) found no increase in nitrogen excretion following the feeding of benzoic acid to a pig maintained

on a pure starch diet. Csonka (3) on the other hand found that on a protein-starch diet benzoic acid did cause an augmented nitrogen output, but by feeding only starch his results were somewhat similar to those of McCollum and Hoagland. Delprat and Whipple (13) found that benzoic acid when administered to fasting dogs caused an increase in urinary nitrogen far in excess of the nitrogen theoretically required for the formation of hippuric acid. They observed, furthermore, that this increased nitrogen excretion could be greatly reduced by feeding dextrin. Epstein and Bookman (14) made a somewhat similar observation on rabbits which they interpreted merely as a manifestation of the sparing action of carbohydrate on protein. Shipley and Sherwin (15) found that moderate doses of benzoic acid did not cause an elevated nitrogen metabolism when given to a human subject who was practically reduced to a stage of endogenous nitrogen metabolism.

The experiments reported in this paper were primarily undertaken with the purpose of determining whether a dog which was amply supplied with carbohydrate could synthesize glycuronic acid without increased nitrogen catabolism. Since benzoic acid was used as the means of producing glycuronic acid, the synthesis of hippuric acid also had to be considered, especially in so far as it pertained to the main problem.

#### EXPERIMENTAL.

All experiments were carried out on female dogs. Glucose and sodium benzoate were dissolved in a 0.5 per cent agar solution and administered by stomach tube. The dose was divided into two portions and the first given in the morning and the second early in the afternoon. Borneol was given in the same way. The dogs were catheterized once a day for the collection of a 24 hour specimen of urine. Dog 12, which received the special fat-carbohydrate diet, was fed once a day and the sodium benzoate incorporated with the food. The same analytical methods were employed as in the previous investigations (1). The urines were examined polariscopically, and, on the days when no benzoic acid was fed, tested for reducing power in order to determine whether the administration of large amounts of glucose such as were fed in these experiments caused alimentary glycosuria. At no time was glucose found in the urine.



## DISCUSSION.

From the results presented in this paper, it can be concluded that the dog is able to synthesize large quantities of glycuronic acid without any marked increased excretion of urinary nitrogen provided the animal is furnished with sufficient carbohydrate. Thus, for example, Dog 2 (Table II) synthesized 5.0 gm. of glycuronic acid with a nitrogen output for that day of only 1.35 gm. as compared to 1.13 gm., the daily average excretion for the 2

TABLE I.

*Study of Effectiveness of a Fat-Carbohydrate Diet in Preventing an Increase in Nitrogen Catabolism Following Ingestion of Benzoic Acid. Means of Establishing That Glycuronic Acid Can Be Derived from Sources Other than Protein.*

Dog 12, weight 6.4 kilos.

Date.	Daily excretion.			Remarks.
	Nitrogen.	Glycuronic acid.	Hippuric acid.	
1926	gm.	gm.	gm.	
June 29	1.22			30 gm. lard fed.
" 30	2.70	2.95		30 " " + 3 gm. sodium benzoate.
July 1	1.65			30 gm. lard.
" 3	1.30	2.86	0.65	Special diet* + 3 gm. sodium benzoate.
" 4	0.80			" " "
" 5	0.97	2.90	0.73	" " + 3 gm. sodium benzoate.
" 6	0.69			" " "
" 7	1.19	2.20	1.03	70 gm. lard + 3 gm. sodium benzoate.

\* The special diet consisted of 41 gm. of starch, 30 gm. of sucrose, and 27 gm. of lard.

previous days when the carbohydrate intake was the same, but no benzoic acid was fed. It is evident that the glycuronic acid could not have been synthesized from protein, and since fat in other experiments failed to prevent benzoic acid from producing an elevated nitrogen metabolism, one has rather definite evidence that glycuronic acid can be derived from the carbohydrate store of the organism. It seems quite probable that the well nourished animal can derive all the glycuronic acid that it is called upon to produce from its store of carbohydrate. This explains why such large quantities of benzoic acid can be fed to dogs for long periods

of time without causing any toxic symptoms. There is no evidence, however, that glucose can go directly to glycuronic acid. On the contrary, the results recorded in Table III lead one to believe that glucose *per se* cannot prevent an increased nitrogen catabolism following the ingestion of benzoic acid. In these

TABLE II.

*Study of Effectiveness of a High Carbohydrate Intake in Preventing Benzoic Acid from Increasing Protein Catabolism. Means of Establishing That the Organism Can Synthesize Glycuronic Acid from Carbohydrate.*

Dog 2, weight 10 kilos. Dog 6, weight 13 kilos.

Date.	Dog No.	Daily excretion.			Remarks.
		Nitro-gen.	Gly-curonic acid.	Hip-puric acid.	
1928		gm.	gm.	gm.	
June 12-14	2	1.16*			100 gm. glucose fed daily.
" 15		1.30	4.71	0	100 " " + 5 gm. sodium benzoate.
" 16		1.05	3.90		100 gm. glucose + 5 gm. borneol.
" 20-21		1.13†			100 " " daily.
" 22		1.35	5.00	0‡	100 " " + 5 gm. sodium benzoate.
" 23	6	1.45	4.50	0.59	50 gm. glucose + 5 gm. sodium benzoate.
" 25		1.06	4.40		50 gm. glucose + 5 gm. borneol.
" 20-21		2.06†			100 " " daily.
" 22		2.16	4.23	0.05	100 " " + 5 gm. sodium benzoate.
" 23		2.03	3.74	1.23	50 gm. glucose + 5 gm. sodium benzoate.
" 25		1.38	3.60		50 gm. glucose + 6 gm. borneol.

\* Average of 3 days.

† Average of 2 days.

‡ 0.97 gm. of free benzoic acid excreted.

particular experiments the dogs were fasted 3<sup>1</sup> days prior to the feeding of benzoic acid in order to reduce the glycogen stores. Although enough glucose was administered with the benzoic acid to form theoretically about ten times as much glycuronic acid as was actually produced, the amount of nitrogen excreted during

this period was markedly higher than it was when no benzoic acid was given. This seems to indicate that glycogen rather than ordinary glucose is the source of glycuronic acid, and that when this supply is insufficient, the organism can synthesize glycuronic acid more readily from protein, or what is more probable, from certain circulating amino acids than from ordinary  $\alpha$ - and  $\beta$ -glucose. Considerable more data, of course, are necessary before one can draw definite conclusions. For the present it is best, perhaps, merely to state that the organism can apparently produce glycuronic acid both from its store of carbohydrate and from protein. This is in harmony with the hypothesis presented in the

TABLE III.

*Effect of Benzoic Acid on Nitrogen Metabolism Following Preliminary Period of Fasting.*

Date.	Dog No.	Average daily excretion.			Remarks.
		Nitro- gen.	Gly- curonic acid.	Hip- puric acid.	
1926		gm.	gm.	gm.	
June 1-3*	6	2.41	4.2	1.22	50 gm. glucose + 4.8 gm. sodium benzoate fed daily.
" 3-6		1.54			50 gm. glucose daily.
" 1-3	2	2.11	5.3	1.16	50 " " + 4.8 gm. sodium benzoate daily.
" 3-6		1.05			50 gm. glucose daily.

\* Both dogs were fasted from May 27 to June 1. Dog 2 was accidentally fed 200 gm. of meat during this period.

previous paper (1) that the precursor of glycuronic acid may be a simple substance, probably a three carbon atom component, which can be derived either from the organism's store of carbohydrate or from the glycogenic amino acids. Further work on the origin of glycuronic acid is in progress.

From the foregoing discussion it is easy to understand the observation of Delprat and Whipple (13) that the increase in the excretion of nitrogen that is induced by feeding benzoic acid to fasting dogs can be prevented by dextrin. Their results are in accord with the present findings except in so far as they consider all of the combined benzoic acid as hippuric acid, whereas, it was

undoubtedly mostly glycuronic acid monobenzoate. It seems very probable that the results obtained by McCollum and Hoagland (12) can also be explained by the conjugation of a considerable portion of the benzoic acid with glycuronic acid. It will be recalled that Csonka (3) found that in some of his experiments on the pig nearly half of the ingested benzoic acid was combined with glycuronic acid. To what extent the formation of glycuronic acid monobenzoate has influenced the results obtained on other animals is hard to estimate since quantitative data are entirely lacking. A rather interesting observation recorded by Lewinski (16) might be mentioned. He noted that the reducing power of the urine which resulted when moderately large doses of benzoic acid were fed to man could be almost entirely prevented if foods rich in glycine were included in the diet. Apparently also in man the partition of the two forms of conjugated benzoic acid is influenced by diet.

Apart from the fact that glycine either free or in the protein molecule can influence the quantity of hippuric acid produced as well as the rate of synthesis (Griffith and Lewis (17)) little is known concerning the other factors that govern the conjugation of benzoic acid. As was stated at the outset, the present research was undertaken primarily for the purpose of determining whether glycuronic acid can be synthesized from carbohydrate. Certain observations that were incidentally made on the formation of hippuric acid can be briefly discussed. On inspecting Table II, it will be found that the administration of large quantities of glucose tends to diminish markedly the production of hippuric acid. In fact, on two occasions no hippuric acid was found. It appears that these synthetic processes follow roughly the law of mass action, and in these particular cases the great excess of carbohydrate effectively prevented the synthesis of hippuric acid. The quantity of glycuronic acid, contrary to what one would expect, was not appreciably increased, but instead, there was a marked rise in the excretion of free benzoic acid.

It hardly seems probable that the limited production of hippuric acid in the dog is due to a lack of glycine. Thus, in some recent unpublished experiments it was found that over twice as much glycine was combined with phenylacetic acid as with benzoic acid under similar conditions. It seems rather certain that all

the glycine that the dog does use for the synthesis of hippuric acid is derived from preformed stores. To what extent other animals can synthesize glycine for the purpose of conjugation is difficult to say. Before this can be answered with certainty, many of the older experiments must be repeated with the better analytical methods which differentiate between hippuric acid and glycuronic acid monobenzoate.

#### SUMMARY.

The production of glycuronic acid in the dog following the ingestion of borneol or sodium benzoate causes little increase in urinary nitrogen provided the animal is furnished with sufficient carbohydrate. This indicates that the organism can produce glycuronic acid from carbohydrate. After a period of fasting, glucose administered with sodium benzoate does not prevent an increased nitrogen catabolism, which makes it appear probable that the precursor of glycuronic acid is derived more readily from glycogen and glycuronic amino acids than from glucose.

The production of hippuric acid in the dog is markedly reduced by the administration of large quantities of glucose with the benzoic acid.

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## THE SUGAR IN URINE AND IN BLOOD.

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### *I. Revision of Folin's Method for the Determination of Sugar in Normal Urine.*

In the application of Folin's<sup>1</sup> new copper reagent and molybdate-phosphate reagent to the determination of sugar in urine, one most unfortunate oversight occurred. No attempt was made to ascertain whether known amounts of glucose added to urine were quantitatively recovered. There was no tangible reason to believe that the new method might be less dependable than the Folin-Berglund process in this respect. F. B. Kingsbury<sup>2</sup> of the Metropolitan Life Insurance Company, found, almost immediately after the method was published, that glucose when added to urine is not recovered by the new process, and Benedict<sup>3</sup> has recently described with considerable detail this unsuspected error in the method.

Benedict's article conveys the impression that the astonishingly low sugar values for normal urine reported in Folin's paper are adequately accounted for by the deficits obtained for added glucose. This interpretation seemed to us improbable, yet the flaw is sufficiently serious so that the method would necessarily have to be abandoned unless the errors involved could be eliminated.

While Lloyd's alkaloidal reagent requires the presence of an acid for the removal of creatinine and similar nitrogenous reducing products, it is not at all necessary that the acid should be a strong one, such as sulfuric acid; even as weak an acid as boric acid is adequate. But the weak acids produce very much the

<sup>1</sup> Folin, O., *J. Biol. Chem.*, 1926, lxxvii, 357.

<sup>2</sup> Private communication.

<sup>3</sup> Benedict, S. R., *J. Biol. Chem.*, 1926, lxxviii, 766.

same effect; they dissolve nearly as much of the Lloyd reagent, setting free large amounts of some calcium salt which destroys the quantitative character of the subsequent reduction with Folin's copper reagent. The effect of oxalic acid is, however, quite different in its action on the Lloyd alkaloidal powder. It is just as effective as any other acid for the removal of creatinine, uric acid, etc. With oxalic acid Lloyd's reagent yields very little of the disturbing calcium-containing material and one gets very slight turbidity on neutralizing the filtrates. When working with glucose solution containing  $\frac{1}{4}$  volume of 0.05 normal oxalic acid, the loss of sugar in the determination on the Lloyd filtrate amounts to from 5 to 7 per cent. In actual urine analysis, the acidity of the urine contributes to the solvent action of the mixture on Lloyd's reagent. The difficulties thus encountered with urine finally led to a very simple and convenient remedy which is equally serviceable with urine and with sugar solutions. The disturbing ingredients which go into the Lloyd filtrates are removed on shaking the filtrate with permutit.

In order to demonstrate the helpful effect of permutit, shake a standard sugar solution, containing  $\frac{1}{4}$  volume of 0.05 normal oxalic acid, with Lloyd's reagent. Shake a part of the filtrate (20 cc.) with about 2 gm. of permutit for 2 to 3 minutes and then make sugar determinations side by side on the two filtrates. The solution which has been through the permutit treatment reduces like a pure sugar solution, whereas the other gives a complex greenish precipitate from which less color is obtained when the molybdate reagent is added. The permutit has substantially the same effect if added directly to Lloyd's suspension before filtering, and shaking another 2 or 3 minutes.

The new process for the determination of sugar in normal urine is therefore as follows:

To 5 cc. of urine in a 50 cc. Erlenmeyer flask add 5 cc. of 0.05 normal oxalic acid, 10 cc. of water, and (last) 1.5 gm. of Lloyd's alkaloidal reagent. Shake gently for 4 minutes and filter through a quantitative filter paper into another small flask containing 2 gm. of permutit; shake with the permutit for 3 minutes. Decant the liquid into a test-tube. It is usually not necessary to filter from the permutit. A very slight turbidity in this solution does not affect the results. This final liquid is also nearly neutral;

but it is probably safest always to add to the sugar tube as many drops of 0.1 normal sodium hydroxide as are indicated by a preliminary titration of the liquid (with phenolphthalein as indicator). To one sugar tube add 2 cc. of the final urine filtrate and to another add 1 cc. plus 1 cc. of water. Add to each the requisite amount of tenth normal sodium hydroxide, if any is called for by the preliminary titration. Transfer 2 cc. of the standard sugar solution to a third sugar tube. To each of the three tubes add 2 cc. of Folin's copper reagent and heat in boiling water for 10 minutes. Cool in running water. Add 2 cc. of molybdate-phosphate reagent, and after about 1 minute, dilute to volume and mix by inverting the tubes three times. Make the color comparison immediately with the unknown which comes nearest in color to that of the standard.

If the standard is set at 20 mm., then 20 divided by the colorimetric reading, and the result multiplied by 80 or 40 depending on whether 1 or 2 cc. of the urine filtrate is used, gives the sugar in mg. per cent or mg. per 100 cc. of urine.

In the case of very weak urines the prescribed dilution of 1 in 4 may give filtrates which have so little sugar that it cannot be determined by the help of the 0.1 mg. standard. In that case it is best to take 10 cc. of urine with 10 cc. of the oxalic solution and omit the addition of water, for the Lloyd-permutit treatment.

In Table I are given the sugar values obtained on Lloyd-permutit filtrates from urine by the Folin-Wu copper reagent and by the new process. The latter method was also applied simultaneously to filtrates containing added glucose. The significance of these figures seems clear. The values obtained by the new process are dependable, and they are still very much lower than the Folin-Berglund values.

We believe that the sugar values given for normal human urine by the new process come very near to being the lowest obtainable by any copper method which is quantitative for glucose.

## *II. The Blood Sugar as Obtained by Different Methods and under Different Conditions.*

The misleading results obtained in sugar determinations on urine by Folin's process were entirely due to the disturbing fac-



tors introduced with Lloyd's alkaloidal reagent. Glucose added directly to diluted and neutralized urine is quantitatively recovered. The flaw in the original method, as applied to urine, has therefore no bearing on its application to blood filtrates.

In the course of a rather extensive series of investigations of blood sugar by means of the process we have, however, discovered another weakness in the method, a weakness which might long have remained undiscovered, but which nevertheless is of considerable importance. As was pointed out in Folin's original communication, the new copper reagent has been adjusted very closely in its range of alkalinity and at this range the available buffer effect is slight. Since the low alkalinity is the essential

TABLE I.  
*Mg. of "Glucose" per 100 Cc. of Urine.*

Original urine.		Original urine plus 40 mg. of glucose per 100 cc.		
Folin-Berglund.	Folin.	Folin.	Theoretical.	Difference.
116	72.5	110	112.5	-2.5
88	61.6	98.4	101.6	-5.2
96.8	55.6	96	95.6	+0.4
72	41.2	82.4	81.2	+1.2
131	116.8	158.4	156.8	+1.6
42	24.6	61.2	64.6	-3.4
54	36.8	74.4	76.8	-2.4

point of the reagent, its composition cannot be materially altered, nor has it been possible to increase the buffer capacity. The specific merit of the reagent lies in the fact that it yields lower blood sugar values than any other copper method and it is only by keeping very close to the lowest permissible degree of alkalinity that the lowest blood sugar figures are obtained. In the course of numerous daily blood sugar determinations made during several months it was soon found that differences between the Folin-Wu and the Folin values were less large than they should have been according to our earlier experience. This led us to try out different copper reagents which had been made according to the same formula. The solutions did not give identical values on hyperglycemic blood filtrates.

These differences were at first ascribed to the possibility that the ingredients, particularly the sodium carbonate, had not been weighed out equally carefully in all cases; but it soon developed that the older solutions invariably gave the higher sugar values on hyperglycemic bloods. It seemed rather incredible that the solutions could get enough alkali from the glass containers to produce any effective change in alkalinity, but to determine this point the newer solutions were all kept in well paraffined bottles. But in the course of about 3 weeks the solutions were again unmistakably stronger than freshly prepared reagents. The cause of the deterioration of the copper reagent was finally found to be due to a spontaneous loss of carbon dioxide. On passing air over the reagent in a cylinder and then into a solution of barium hydroxide, visible barium carbonate precipitates were obtained almost immediately, and on passing air through a freshly prepared reagent for an hour, we obtained a solution which gave the same sugar values as the reagent of Folin and Wu.

In the light of this experience the only safe rule is to keep Folin's copper reagent in small, well filled, tightly corked bottles, and not to depend on the bottle in daily use and frequently opened for more than 7 to 10 days.

In the discussion given above, we have rather overemphasized the danger of deterioration of the copper reagent. As a matter of fact, reagents several months old and kept in large containers will still give less sugar with hyperglycemic bloods than are obtained with the Folin-Wu copper solution. But the true minimum sugar values cannot be obtained with such solutions.

The thought will doubtless occur to many that, if the reagent can thus lose significant amounts of carbon dioxide spontaneously, it must lose a great deal during the actual heating with the sugar, and that it therefore must necessarily become progressively more alkaline during the 10 minute heating period. However correct theoretically such an interpretation may be, the fact is that in the matter of agreement between duplicate determinations and in the matter of proportionality between different amounts of sugar the new copper reagent is fully as dependable as the older copper solution of Folin and Wu.

From the stated considerations it follows, however, that the prescribed directions must be followed in the making of the de-

terminations, for the conditions are as much a part of the method as is the formula for the making of the reagent.

It seems best to specify the conditions to be observed in making blood sugar determinations by Folin's process.

1. The blood filtrates must be neutralized to a *permanent* pink color with phenolphthalein. Correctly made blood filtrates are so nearly neutral that 10 cc. of filtrate require only 0.2 cc. of tenth normal sodium hydroxide to give a permanent pink color. But if too much oxalate has been added to the blood, the filtrates are invariably more acid and such filtrates will usually be found to contain demonstrable amounts of tungstic acid. When 2 cc. of the filtrate are added to 1 cc. of concentrated sulfuric acid no visible turbidity should occur in the course of an hour. Blood filtrates which give a positive test for tungstic acid, by this test, and which at the same time contain over 200 mg. per cent of sugar may give results that are from 2 to 5 per cent too low. As the described test for tungstic acid is by no means a sensitive one, a positive result with it certainly means that the precipitation of the blood proteins has not been made under the best conditions. A positive test indicates (a) that too much anticoagulant has been added or (b) that the sodium tungstate and  $\frac{2}{3}$  normal sulfuric acid are not adjusted or (c) that the sodium tungstate contains more than traces of para-tungstate.

2. Only copper solutions which have had no chance to lose much carbon dioxide should be used. This object is attained by keeping the copper reagent in small, well filled, and tightly corked bottles.

3. The time of boiling, 10 minutes, must be adhered to. The water bath should be vigorously boiling when the sugar tubes are immersed, and kept vigorously boiling for the prescribed period.

We have the impression that a rapidly boiling water bath is distinctly more effective than one which is barely boiling. This may be due to the fact that the sugar tubes are more or less jarred by the rapidly boiling water.

4. As soon as the tubes have been cooled in a beaker of running water for 1 to 5 minutes, add the molybdate-phosphate reagent and make the color comparison immediately.

For the fine determinations required when very little sugar is

present the molybdate solution should be substantially colorless. The solution remains colorless if not exposed to too much light; hence it is best to keep the stock solution in the dark and to keep only a portion of it in daily use.

5. When a series of sugar determinations is being read against the same standard it is best to move the plunger out of the standard solution and then bring it back to 20 mm. for each new determination. If this is not done, fine CO<sub>2</sub> bubbles may collect under the plunger of the standard and the result will be too high readings and too low sugar values for the later determinations.

6. The blood filtrate actually used in the sugar tube should not contain much more than 0.5 mg. of sugar because most of the copper in the reagent is used up by this amount of sugar.

When these precautions are observed, the new copper reagent will give reliable, correct values for the blood sugar. By correct values we mean the *lowest* values obtainable under conditions which will yield also a quantitative recovery of glucose.

The figures in Table II are given to show that glucose added to blood is quantitatively recovered. In these experiments we also employed the Folin-Wu copper reagent, but as it has long been recognized that added glucose is recovered by this process we omit the Folin-Wu figures.

Concerning the application of the Folin-Wu method it should be stated that inasmuch as we needed to get the most accurate values which this method could give, we have used a table of corrections for the somewhat imperfect degree of proportionality which one obtains when the standard and the unknown are too far apart. These deviations from true proportionality are not large when the new molybdate reagent is used, but it seemed best, nevertheless, to correct for them.

In Table III is recorded a series of blood sugar values determined by the copper tartrate reagents of Folin and Wu and of Folin. The bloods were all taken from Dr. Joslin's diabetic patients. While there is no sharp regularity in the differences between the two sets of sugar values, it is clear that these differences tend to be greater the higher the level of the blood sugar. These results clearly show that the hyperglycemia of diabetic blood is not all due to an accumulation of glucose. From 15 to nearly 25 per cent of the sugar in diabetic blood *must* be represented by something else than glucose and since more or less of the same

unknown sugar materials *may* be included in the Folin sugar values one must be prepared for the possibility that the unknown sugars are present in still larger proportions.

Fermentation by yeast has been considered to furnish the most specific principle for the determination of reducing sugar, and of

TABLE II.  
*Showing that Glucose Added to Blood Is Quantitatively Recovered by Folin's Method.*

	Error.	
	mg.	mg.
1. Sugar in original blood.....	91	
Same plus 50 mg. of glucose.....	143	+2
“ 100 “ “ “ .....	189	-2
2. Sugar in original blood.....	63	
Same plus 100 mg. of glucose.....	165	+2
3. Sugar in original blood.....	49	
Same plus 250 mg. of glucose.....	299	0
4. Sugar in original blood.....	242	
Same plus 100 mg. of glucose.....	342	0
5. Sugar in original blood.....	234	
Same plus 100 mg. of glucose.....	328	-6
6. Sugar in original blood.....	316	
Same plus 100 mg. of glucose.....	408	
7. Sugar in original blood.....	376	
Same plus 100 mg. of glucose.....	480	+4
8. Sugar in fresh sheep blood.....	30	
Same plus 200 mg. of glucose.....	228	-2
“ 400 “ “ “ .....	420	-10

late a number of investigators have attempted to make use of the fermentation process for the determination of the blood sugar. These investigators have applied the yeast treatment directly to blood and have determined the sugar in the blood before and after fermentation. We have adopted a seemingly more con-

TABLE III.  
*Blood Sugar in Mg. Per Cent (Diabetic Blood).*

No.	Folin-Wu.	Folin.	Difference.
1	107	93	14
2	114	101	13
3	125	101	24
4	127	109	18
5	127	109	18
6	140	115	25
7	150	118	32
8	141	121	20
9	142	125	17
10	149	129	20
11	155	134	21
12	170	133	37
13	152	132	20
14	179	138	41
15	169	143	26
16	177	148	29
17	178	157	21
18	182	160	22
19	183	165	18
20	196	176	20
21	215	183	32
22	211	187	24
23	252	190	62
24	244	200	44
25	233	204	29
26	263	230	33
27	250	220	30
28	276	220	56
29	280	222	58
30	280	230	50
31	280	229	57
32	256	230	26
33	280	238	42
34	308	256	52
35	329	282	47

venient method for the determination of the fermentable sugar. Yeast works just as expeditiously in the tungstic acid blood filtrates of Folin and Wu as in pure sugar solutions.

The removal of the fermentable sugar from these filtrates seemed to us particularly interesting in connection with our

determinations of the sugar by two reduction methods which give distinctly different results for the blood sugar. If yeast removes only glucose, or glucose plus other sugars with reducing power equal to that of glucose, or greater, then the amount of fermentable sugar should be practically independent of the copper method by which the sugar is determined before and after fermentation. On the other hand, if yeast also takes from the blood sugars with weaker reducing properties than those of glucose, then the amount of fermentable sugar obtained should depend in part on the copper method used and, in this particular case, the fermentable sugar determined by the method of Folin and Wu should be greater than that found by the new copper method. This is a new problem, of course, only in relation to the fact that our copper methods yield such different values for the blood sugar.

In Table IV are given the blood sugar values obtained by Folin's copper process and the *fermentable sugar* as obtained by the copper solutions of Folin and Wu and of Folin.

From the first two sets of figures in the table it will be seen that the sugar obtained directly by the new copper method is actually smaller than the fermentable sugar as determined by the copper method of Folin and Wu. The fermentable sugar may be the same or even a little lower than the values obtained by direct reduction where the blood sugar is normal, but as the higher values are reached the fermentable sugar increases much faster than the Folin copper values, until, at the bottom of the table, we get up to 30 mg. per cent more sugar by fermentation than by direct reduction with Folin's copper reagent.

The fermentable sugar obtained by Folin's copper reagent, as given in the last column, is much lower than the corresponding values found by the Folin-Wu copper reagent, especially in bloods having more than 250 mg. per cent of sugar. On the other hand, these fermentable sugar values are only a trifle higher than those obtained by direct reduction of the same copper reagent. These differences are somewhat larger, however, than is shown by the figures, because the fermentation values are given exactly as obtained, without making any deduction for the "blanks" which are obtained on suspending yeast in water. This blank is equivalent to from 3 to 5 mg. per cent of sugar.

TABLE IV.

*Showing that "Fermentable Sugar" Depends on Reduction Method Employed and that Folin's Reduction Values on Diabetic Blood Are Lower than the Fermentable Sugar Obtained by the Copper Method of Folin and Wu.*  
*Blood Sugar in Mg. Per Cent (Diabetic Blood).*

No.	Fermentable sugar, Folin-Wu.	Folin.	Fermentable sugar, Folin.
1	87	93	85
2	95	101	93
3	103	101	94
4	102	109	95
5	107	109	100
6	118	115	107
7	128	118	107
8	125	121	112
9	126	125	118
10	128	129	121
11	135	134	122
12	148	133	123
13	134	132	124
14	157	138	130
15	147	143	133
16	158	148	141
17	158	157	149
18	161	160	152
19	168	165	158
20	180	176	168
21	193	183	176
22	196	187	179
23	237	190	182
24	221	200	187
25	210	204	195
26	237	230	215
27	233	220	212
28	257	220	213
29	260	222	214
30	259	230	218
31	260	229	222
32	236	230	220
33	256	238	225
34	283	256	246
35	312	282	276



The fermentable sugar values recorded in Table IV were obtained by determining the reduction before and after treatment with yeast. To complete the record the latter values must also be given. As these non-fermentable, or rest reduction, values are of considerable interest they are given separately in Table V. The non-fermentable sugar or reduction values are quite independent of the total reducing sugar originally present in the tungstic acid blood filtrates. Whether the blood contained 100 or 300 mg. per cent of sugar, the non-fermentable fraction amounts to about 10 mg. per cent when the determination is made by Folin's copper reagent, and to about twice that amount, 20 mg. per cent, when the Folin-Wu copper reagent is used.

These are uncorrected values. If one were to subtract the blank reduction obtainable from yeast and water the unfermentable sugar obtained by the Folin process would sink to about 5 or 6 mg. per cent. This figure, 5 or 6 mg. of sugar per 100 cc. of blood, represents then the average non-fermentable reducing material which is included in the sugar determinations made with Folin's new copper tartrate reagent. It is clear, therefore, that except in certain bloods, notably nephritic blood, nitrogenous materials contribute extremely little to our values for the blood sugar.

In view of the uncertainty as to the chemical character of a part of the blood sugar, our conclusion that not more than 5 to 6 mg. per cent of the same can be due to nitrogenous products is manifestly of some importance.

A preliminary brief report of some other experiments bearing on reduction products other than sugars may be given here.

One more or less predominant aim in the development of new sugar methods has been to prevent reducing nitrogenous products from taking part in the reaction. The success achieved has not been particularly good except on the basis of a preliminary removal of the nitrogenous products.

For more than a year we have had available in this laboratory copper reagents which do differentiate between reducing sugars and reducing nitrogenous products. These reagents, however, are negative for the sugars and positive with the nitrogenous products. The main principle in the preparation of these reagents is the use of ammonia plus ammonium salts for the produc-

TABLE V.

*Showing that the Non-Fermentable "Sugar" (Rest Reduction) Is Independent of Original Sugar Level of the Blood and Is Approximately Constant for Each Copper Method.*

*Non-Fermentable Sugar "Rest Reduction" (Diabetic Blood) in Mg. Per Cent.*

No.	Folin-Wu.	Folin.
1	20	8
2	19	8
3	22	7
4	25	14
5	20	9
6	22	8
7	22	11
8	16	9
9	16	7
10	21	8
11	20	12
12	22	10
13	18	8
14	22	8
15	22	10
16	19	7
17	20	8
18	21	8
19	15	7
20	16	8
21	22	7
22	15	8
23	15	8
24	23	13
25	23	9
26	26	15
27	17	8
28	19	7
29	20	8
30	21	12
31	20	7
32	20	10
33	24	13
34	25	10
35	17	6

tion of the required degree of alkalinity, together with tartrates or citrates, as a supplementary means of holding the cupric hydrate in solution. One such reagent is prepared as follows:

Dissolve 100 gm. of ammonium sulfate in about 400 cc. of water and filter into a volumetric liter flask. To this solution add 100 cc. of 10 per cent sodium hydroxide, 12 gm. of sodium tartrate, and finally a solution of 5 gm. of copper sulfate. Dilute to volume and mix.

This reagent gives excellent reductions with creatinine, uric acid, allantoin, etc., yet gives not even a trace of color with the standard glucose solutions. On adding 2 cc. of this reagent to 2 cc. of blood filtrate in a sugar tube and heating 10 minutes, then cooling and adding 2 cc. of the molybdate reagent, one gets so little color that it is scarcely practicable to try to read it quantitatively. These nearly negative reactions indicate that there are no unknown nitrogenous products in the tungstic acid blood filtrates which contribute materially to the blood sugar values of normal or diabetic blood.<sup>4</sup>

A brief description of the fermentation process which we have employed must now be given.

To 20 cc. of the blood filtrate (which must not contain toluene, sodium fluoride, or other preservative) is added one-quarter of a *fresh* Fleischmann yeast cake (3.25 gm.). The mixture is shaken vigorously so as to produce a uniform suspension and the flask is then immersed in water at 37–40°C. and after some shaking to promote the rise in temperature is left in the water bath for 7 to 8 minutes. This short fermentation is quite adequate for the removal of the fermentable sugar, and prolonged fermentations are undesirable in that they tend to increase the blank and give more residual reduction than does the shorter period. At the end of the fermentation period a small teaspoonful (1.75 gm.) of kaolin is added; the mixture is again shaken, cooled, and filtered through a quantitative filter paper. This simple, rapid, process for removing the fermentable sugar gives perfectly dependable, uniform results both with blood filtrates and with correspondingly dilute sugar solutions.

For the determination of the minute amounts of color-giving materials

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<sup>4</sup> In a recent contribution by H. F. Holden, it is suggested that from 10 to 15 per cent of the sugar found in "biological fluids" may be due directly or indirectly to amino acids. We certainly do not think that any such proportion is contributed by the nitrogenous products of normal blood filtrates when the sugar is determined by Folin's new process. Holden, H. F., *Biochem. J.*, 1926, xx, 263.

in the filtrate it is, of course, necessary to use very dilute standard sugar solutions (0.025 mg. per cc.) and it is also necessary so to turn the colorimetric reflector that only a little light passes up through the colorimetric cups. It is also important that the molybdate reagent should be substantially colorless. With these precautions, however, the sugar values of the fermented filtrates can be determined by our reagents with an astonishing degree of accuracy.

In view of the criticisms recently advanced by Ege<sup>5</sup> against the short fermentation period used by Van Slyke and Hiller<sup>6</sup> we recognize that inasmuch as our fermentation period is even shorter than Van Slyke's (8 minutes instead of 20) our process might come in for even stronger criticisms on the score of incomplete fermentations. We are inclined to think, however, that by using very short fermentation periods one obtains not only an obviously convenient analytic method, but also the maximum fermentation values, at least in relation to our colorimetric methods for estimating the rest reduction.

In this connection it may be mentioned that the rest reductions which are obtained after prolonged yeast treatment are not only larger, but less uniform than those obtained at the end of short fermentation periods. The same phenomenon is obtained with pure glucose solutions. The reducing products given off by yeast are not predominantly made up of sugars; they contain reducing nitrogenous products, as can be shown by means of the ammoniacal copper solution described above.

It may be mentioned in passing that the rest reductions obtained in our fermentation process are substantially identical with the rest reductions obtained by glycolysis. This statement is based on scores of glycolysis experiments made in this laboratory during the past season. The introduction of yeast is therefore practically superfluous, if one is willing to wait 24 hours before making the final determination. The values so obtained will be about 7 mg. per cent by the Folin process and about twice as large by the Folin-Wu method, and this rest reduction value, like that obtained with yeast, is independent of the original sugar content of the blood.

For the sake of brevity and without pretense of accuracy, we

<sup>5</sup> Ege, R., *J. Biol. Chem.*, 1926, lxviii, 317.

<sup>6</sup> Hiller, A., Linder, G. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1925, lxiv, 625. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, 1926, lxviii, 323.

may refer to differences represented by the blood sugar values of Folin-Wu and Folin as non-glucose sugar. That these differences actually represent a large fraction of fermentable sugar materials other than glucose is clearly shown by the fact that the (uncorrected) Folin-Wu rest reductions are almost uniformly only 12 mg. higher than those of Folin's method, while the non-glucose sugar shown in Table II varies from 13 mg. to over 50 mg. Where the difference is 50 mg. we should thus have at least 35 mg. per cent of fermentable non-glucose sugar which is not determined by Folin's process. It seems to us extremely probable that this fermentable non-glucose sugar of blood must have some significance in relation to the intermediate metabolism of carbohydrates. From this point of view there follows one practical conclusion.

In the future development of new analytical methods for blood sugar, the aim must be to find processes that give either lower values than those of Folin's method or higher values than those given by the method of Folin and Wu. Analytical processes which give intermediate values are less likely to furnish new information of permanent value. The fermentable sugar obtained by the help of Folin's copper method evidently comes the nearest to the probable true glucose content of blood and the same copper method alone gives values which are not so very far away from those same glucose values.

We have made a number of experiments calculated to elucidate further the variations in the non-glucose sugar in other cases than diabetes.

As was to be expected the non-glucose sugar is not less abundant in the plasma than in the corpuscles. This fact is indicated by the sugar determinations recorded in Table VI. This point is not without significance from one point of view. It practically excludes the otherwise plausible idea that our non-glucose sugar might be maltose. According to Folin and Berglund<sup>7</sup> the plasma sugar is diminished rather than increased by hydrolysis, whereas the sugar of the corpuscles is nearly always increased by hydrolysis.

The most convincing indication that we have yet obtained in

<sup>7</sup> Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, 213.

favor of the view that the non-glucose originates within the organism, and in fact represents some unknown phase of the carbohydrate metabolism, is obtained from the administration of insulin.

The data bearing on this point are given in Table VII. Fasting, normal persons, mostly first year medical students, were given a small dose of insulin, 9 or 10 units, in the morning, and the sugar was then determined at half hour intervals. In five of the six subjects investigated, the fall of the non-glucose sugar was practically just as sharp and decisive as the drop of the total sugar. In most of the cases, the fall of the non-glucose sugar

TABLE VI.

*Showing that the Non-Glucose Sugar Is No Less Abundant in Plasma than in Corpuscles.*

*Blood Sugar (Mg. Per Cent).*

	Folin-Wu.	Folin.	Difference.
Whole blood.....	97	83	14
Plasma.....	101	81	20
Whole blood.....	59	51	8
Plasma.....	55	48	7
Whole blood.....	93	83	10
Plasma.....	93	81	12
Whole blood.....	174	157	17
Plasma.....	184	167	17

was even more marked. The total sugar fell to an average of 60 to 65 mg. per cent, while the non-glucose sugar fell from an average of 15 mg. per cent to about 6 or 7 mg. per cent. In one case, the non-glucose sugar fell from 19 to 3 mg. per cent, and in another from 14 to 4 mg. per cent.

The effect of adrenalin on the non-glucose blood sugar is not nearly so clear cut as is the effect of insulin. It was thought that since the administration of adrenalin produces a rapid mobilization of blood sugar and since this accumulating blood sugar should come more or less directly from glucogen, therefore, the extra blood sugar thus produced should be glucose. If the facts corresponded to this interpretation, we should get a rapid increase

in the total blood sugar, while the non-glucose sugar should remain stationary. The effects of adrenalin are, however, not quite so simple. The adrenalin undoubtedly produces a marked increase in the rate of metabolism. These accelerated oxidation processes might thus even reduce the level of the non-glucose

TABLE VII.

*Showing that Insulin Injection Reduces the Non-Glucose Sugar of Blood.  
Blood Sugar in Mg. Per Cent.*

Subject.	Method.	Before insulin.	After insulin.			
			½ hr.	1 hr.	1½ hrs.	2 hrs.
A.S-g.	Folin-Wu.	113	86	58	60	59
	Folin.	94	56	50	55	56
	Difference.	19	30	8	5	3
W-r.	Folin-Wu.	88	74	61	71	80
	Folin.	74	62	57	67	67
	Difference.	14	12	4	4	13
W.F-s.	Folin-Wu.	99	58	67	68	77
	Folin.	82	52	60	61	68
	Difference.	17	6	7	7	9
H.P-s	Folin-Wu.	97	81	55	56	63
	Folin.	83	70	48	49	53
	Difference.	14	11	7	7	10
T.W-r.	Folin-Wu.	90	64	65	63	70
	Folin.	77	53	53	52	54
	Difference.	13	11	12	11	16
T.W-n.	Folin-Wu.	97	87	76	77	72
	Folin.	86	76	69	70	66
	Difference.	11	11	7	7	6

blood sugar while the blood glucose is going up. Several of our experiments with adrenalin, notably the first four recorded in Table VIII are in good agreement with this latter point of view. In these we find that the non-glucose sugar has actually fallen distinctly below the initial level of 11 or 12 mg. per cent, while the total blood sugar has risen from the ordinary fasting levels

TABLE VIII.

*Illustrating Effect of Adrenalin on Total Blood Sugar and Non-Glucose Sugar.  
Blood Sugar in Mg. Per Cent.*

Subject.	Method.	Before adrenalin.	After adrenalin.			
			$\frac{1}{2}$ hr.	1 hr.	1½ hrs.	2 hrs.
A.S-g.	Folin-Wu.	108	174	182	167	127
	Folin.	96	167	177	161	125
	Difference.	12	7	5	6	2
M.L-n.	Folin-Wu.	91	164	184	174	131
	Folin.	80	158	182	166	119
	Difference.	11	6	2	8	12
H.P-s.	Folin-Wu.	96	169	188	165	139
	Folin.	82	163	182	157	129
	Difference.	14	6	6	8	10
G.R-y.	Folin-Wu.	98	162	176	141	109
	Folin.	87	161	172	134	101
	Difference.	11	1	4	7	8
F.F-l.	Folin-Wu.	101	125	157	165	145
	Folin.	92	115	154	161	143
	Difference.	9	10	3	4	2
T.F-s.	Folin-Wu.	91	178	212	144	85
	Folin.	80	168	195	129	71
	Difference.	11	10	17	15	14
L.S-r.	Folin-Wu.	112	118	134	131	107
	Folin.	103	118	130	129	98
	Difference.	9	0	4	2	9
S-n.	Folin-Wu.	102	225	183	177	
	Folin.	85	185	157	152	
	Difference.	17	40	26	25	
H.W-e.	Folin-Wu.	106	180	189	171	
	Folin.	87	162	165	167	
	Difference.	12	18	24	4	

up to 170 mg. per cent and over. In most of the other experiments the non-glucose sugar remains approximately stationary or has risen by a few mg. per cent. In one case where the total



blood sugar rose from 102 to 225 mg. per cent, the non-glucose sugar rose from 17 to 40 mg. per cent for a brief period.

The only conclusion that can be drawn safely on the basis of these experiments is that the injection of moderate doses (0.8 to 1 mg.) of adrenalin to normal fasting persons increases the total blood sugar relatively much faster than it increases the non-glucose sugar.

Since the non-glucose sugar of blood can be influenced both by insulin and by adrenalin injections, one naturally would inquire whether pure glucose taken by mouth is found in the blood only as glucose or whether a part of it is demonstrably converted into non-glucose sugar. Only two experiments can be given on

TABLE IX.

*Showing that the Non-Glucose Sugar of Blood Is Not Increased by Giving Glucose.*

*Blood Sugar in Mg. Per Cent.*

Subject.	Method.	Before glucose.	After taking 50 to 75 gm. glucose.			
			½ hr.	1 hr.	1½ hrs.	2½ hrs.
A.S-g.	Folin-Wu.	104	158	152		74
	Folin.	92	144	139		66
	Difference.	12	14	13		8
S-n.	Folin-Wu.	96	136	162	167	122
	Folin.	83	124	150	155	109
	Difference.	13	12	12	12	13

this point, but they seem to be fairly conclusive. The absorbed extra sugar in the blood is there as glucose according to the figures recorded in Table IX.

In some of our experiments with adrenalin, as well as with glucose ingestions, the sugar was determined both before and after fermentation. In order not to make the tabular material of this paper too unwieldy, we record here only a few non-fermentable sugar values as given by Folin's method. The first two sets of values in Table X are taken from the glucose experiments; the others represent adrenalin hyperglycemia.

From the figures of Table X the reader will see that the rest reduction, the non-fermentable "sugar" of blood, remains prac-

tically constant, while the total blood sugar, or the sugar as determined by Folin's process, rises and again returns to nearly normal levels. In this respect, experimental hyperglycemia gives the same picture as is obtained from diabetic bloods with very high sugar levels. Incidentally, the figures recorded in Table X may serve to show that the fermentation process described in this paper gives very uniform results.

TABLE X.

*Showing that the Non-Fermentable "Sugar" of Blood Is Not Influenced by Experimental Hyperglycemia.  
Rest Reduction of Blood in Mg. Per Cent.*

Rise in blood sugar.	Before hyperglycemia	During hyperglycemia.			
92-144	8	6	7	7	
83-155	8	7	6	7	10
72-150	11	9	7	8	7
81-135	10	10	8	8	
72-143	10	8	8	8	8
79-123	8	10	9	9	9

## RÉSUMÉ.

1. A revised form of Folin's copper method for the determination of sugar in normal urine is described. This method is believed to give dependable values, and the values so obtained are much lower than the sugar values obtained by the method of Folin and Berglund. These values are also believed to be lower than those obtained by any other known copper method.

2. Folin's method for the determination of sugar in blood has been subjected to a critical study and if certain given precautions are observed the method gives dependable values. The sugar values for blood obtained by this process are believed to come nearer to the glucose content of blood than the values obtained by any other known copper method.

3. A simple, convenient, and dependable fermentation method for the determination of sugar in blood is described.

4. The amount of fermentable sugar obtained depends on the copper method employed for the determination of the sugar before and after fermentation. The lowest values for the fermentable sugar are obtained when Folin's copper method is used.

5. Experimental evidence is given showing that the blood sugar contains fermentable sugar other than glucose. This unknown fermentable sugar is not maltose or any other di- or polysaccharide. The unknown sugar is presumably produced within the organism and represents some phase of the intermediary metabolism of carbohydrates.

6. In response to insulin injections the non-glucose sugar goes down. With adrenalin injections it may go either up or down. It is not influenced by oral administrations of pure glucose.

The non-glucose sugar is most abundant in diabetics with high blood sugar.

7. The non-fermentable reducing material in blood is relatively quite constant and therefore does not vary with the sugar level of the blood, either in normal persons or in diabetic ones. The non-fermentable "sugar" amounts on the average to only about 5 or 6 mg. per 100 cc. of blood, with Folin's copper method. It is larger with the Folin-Wu method.

8. The reducing values of the nitrogenous products in the tungstic acid blood filtrates are insignificant (except in nephritic bloods).

9. A preliminary description is given of another class of alkaline copper reagents which are reduced by the ordinary nitrogenous products, with distinct reducing properties, but are unaffected by reducing sugars.

## THE OSMOTIC PRESSURE OF HEMOGLOBIN AND OF BASE BOUND BY HEMOGLOBIN.

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Adair (1) has recently measured the osmotic pressure of pure dialyzed hemoglobin. He found the following

$$p = \frac{10.21}{4} \left( \frac{C}{1 - 0.015 C} \right)$$

where  $p$  = osmotic pressure in mm. of Hg,

$C$  = gm. of Hb per 100 cc. of solvent.

He also observed (2) the osmotic pressure in solutions of pure dialyzed hemoglobin to which NaOH had been added and from the observed osmotic pressure compared with the osmotic pressure calculated on the assumption that all the BHb was fully ionized he concluded that the ionization of the sodium hemoglobin appeared to be about  $50 \pm 15$  per cent.

The development by Stadie and Ross (3) of a method for preparing quickly a crystalline preparation of hemoglobin relatively free from electrolyte has facilitated the experimental test of the influence of added salt upon the osmotic pressure of hemoglobin across a collodion membrane permeable to salt but not to hemoglobin.

The aspect of the problem which we have studied is that in which enough NaOH has been added to the oxygenated hemoglobin to give about 1.5 m.-eq. of base bound per mm of  $O_2$  capacity. Under these conditions the pH in the hemoglobin solution

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would be from the titration curves of Hastings, Van Slyke, Neill, Heidelberger, and Harington (4) about 7.2 to 7.3.

In this paper we use the following abbreviations.

$\pi$  = osmotic pressure across the membrane. This is expressed either in mm. of Hg ( $\pi$ mm) or in millimols of undissociated non-diffusible solute per kilo of water required in ideal solution to give that osmotic pressure ( $\pi$ mM). The relationship between the units is

$$\frac{\pi\text{mm}}{760} = \frac{\pi\text{mM}}{1000} \times 22.4 \times \frac{T}{273}$$

( ) indicates concentration per liter of solution.

[ ] indicates concentration per kilo of water.

Hb = hemoglobin, concentration expressed in millimols of oxygen capacity.

B = total base, concentrations in milli-equivalents.

A = total diffusible anion, concentrations in milli-equivalents.

BHb = base bound by hemoglobin, concentrations in milli-equivalents.

Cl = total chloride, concentrations in milli-equivalents.

Subscript <sub>i</sub> = inside the collodion sac, in the hemoglobin solution.

Subscript <sub>o</sub> = outside the collodion sac, in the hemoglobin-free dialyzate.

$E_{\text{mem}}$  = potential across the collodion membrane.

$\pi_{\text{Hb}}$  and  $\pi_{\text{BHb}}$  are used to indicate the fraction of the total osmotic pressure attributed to hemoglobin and to base bound by hemoglobin in the theoretical summation of the osmotic effects.

Following the treatment employed by Van Slyke, Wu, and McLean (5), assuming an ideal solution with complete dissociation of salts and of base bound by hemoglobin and assuming that hemoglobin can be treated as if in molecular solution, the osmotic pressure across a membrane impermeable only to hemoglobin is

$$\begin{aligned}\pi\text{mM} &= [\text{B}]_i + [\text{A}]_i + [\text{Hb}] - ([\text{B}]_o + [\text{A}]_o) \\ &= 2 [\text{A}]_i + [\text{BHb}]_i + [\text{Hb}] - 2 [\text{B}]_o\end{aligned}$$

For the same solution the Donnan equilibrium requires

$$[\text{B}]_i [\text{A}]_i = [\text{B}]_o [\text{A}]_o = [\text{B}]_o^2$$

For the potential across the membrane at 23°C.

$$\frac{E_{\text{mem}}}{0.0586} = \log \frac{\alpha_{\text{B}_i}}{\alpha_{\text{B}_o}} = \log \frac{\alpha_{\text{A}_o}}{\alpha_{\text{A}_i}}$$

where  $\alpha$  = activity of the ions designated in subscript. In dilute

solutions as the activity coefficients approach unity this relationship approaches the following expressed in concentration terms

$$\frac{E_{\text{mem}}}{0.0586} = \log \frac{[B]_i}{[B]_o} = \log \frac{[A]_o}{[A]_i}$$

From these equations the effect upon  $\pi$  and  $E_{\text{mem}}$  of adding a diffusible salt such as NaCl to a given amount of isoelectric hemoglobin and of NaOH can be calculated for the case of ideal solution. Values are plotted in Fig. 1 for the case where  $[\text{Hb}] =$

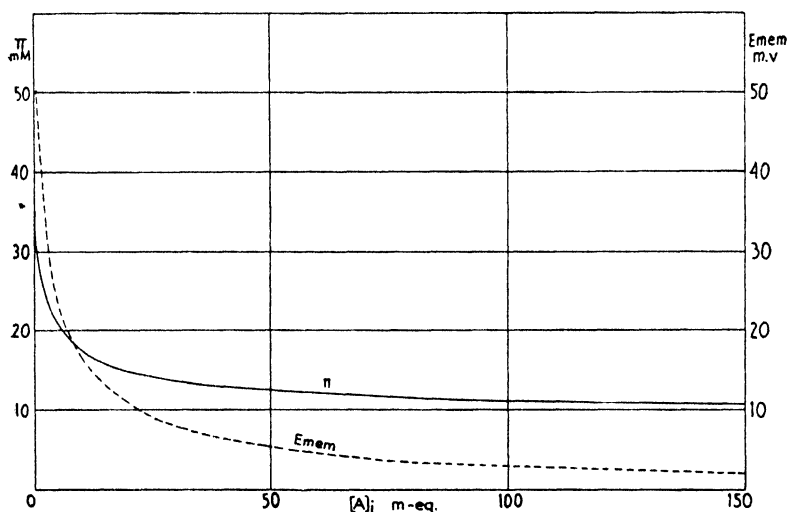


FIG. 1. Osmotic pressure  $\pi$  mm and membrane potential  $E_{\text{mem}}$  in millivolts plotted against  $[A]_i$  for case of hemoglobin solution with  $[\text{Hb}] = 10$  mm and  $[\text{BHb}] = 25$  m.-eq.

10 mm and  $[\text{BHb}] = 25$  m.-eq. As NaCl is increased and hence  $[A]_i$  increases from 0 to 100 m.-eq. the value of  $\pi$  mm falls from the sum  $[\text{Hb}] + [\text{BHb}]$  toward the value  $[\text{Hb}]$ . For  $[A]_i = 100$  m.-eq.,  $\pi$  mm is already within about 1 mm of the value  $[\text{Hb}]$ . If  $[\text{BHb}]$  is less than 25 m.-eq. then  $\pi$  mm will still exceed the value  $[\text{Hb}]$  but, for any given value of  $[A]_i$ , by a smaller amount.

The value of  $E_{\text{mem}}$  as  $[A]_i$  is increased from 0 to 100 m.-eq. falls from the value determined by the distribution of  $[\text{B}^+]$  and of  $[\text{OH}^-]$  toward zero as the diffusible ions are increased. When  $[A]_i = 100$  m.-eq.,  $E_{\text{mem}}$  is very small, about 3 millivolts. When

$[A]_i = 10$  m.-eq., however,  $E_{mem}$  is 16 millivolts. When  $[BHb]$  is less than 25 m.-eq.,  $E_{mem}$  will be larger for any given value of  $[A]_i$  until the zone is reached ( $[B]_o$  less than 1 m.-eq.) where  $E_{mem}$  is determined in large part by the distribution of  $[B^+]$  and  $[OH^-]$ . From these considerations it follows that the measurement of the osmotic pressure of such a solution of hemoglobin in solution of 100 mm  $[NaCl]$  should give approximately the osmotic pressure of the hemoglobin. The osmotic pressure in a solution of very low salt content should give approximately the osmotic pressure of the hemoglobin and of the base bound by hemoglobin. The potential across the membrane of such a hemoglobin solution in a 100 mm  $[NaCl]$  solution should be theoretically only about 3 millivolts. However with reduction in the salt content easily measurable membrane potentials should be obtained.

Our experiments were designed to test these relationships (1) by the measurement of the osmotic pressure and potential across the membrane of an alkaline hemoglobin solution as free of other anion as we could secure, in equilibrium across a collodion membrane with its dialysate into water and (2) by the measurement of the osmotic pressure of similar solution containing, however about 100 m.-eq. of  $NaCl$ .

### *Methods.*

Hemoglobin was prepared from oxalated horse blood by the electro-dialysis method of Stadie and Ross (3). The blood cells were washed three times with 2 per cent  $NaCl$  solution, diluted with water to a hemoglobin concentration of about 15 mm, and electro-dialyzed for 3 to 4 hours. The crystals obtained were washed three times with one-fifth their volume of chilled distilled water ( $4^\circ C.$ ). To 65 cc. of washed crystals were added 40 cc. of distilled water and 37 cc. of  $N/10$   $NaOH$  prepared from concentrated solution of  $NaOH$  and containing less than 1 volume per cent of  $CO_2$ . The hemoglobin was stirred in the open air to facilitate solution for 15 minutes, filtered through gauze, and diluted to 150 cc. This hemoglobin solution was used inside the sacs with distilled water outside in the experiments designated "without added salt." In the experiments designated "with added salt," dry  $NaCl$  was added to this hemoglobin solution in amount sufficient to make approximately 0.1 M  $NaCl$ ; this salted hemoglobin solution was used inside the sacs with 0.1 M  $NaCl$  solution in water outside the sac.

Diffusion equilibrium was established in collodion sacs prepared and fitted as described by Northrop and Kunitz (6). The sacs were tested against a pressure of 200 mm. of Hg. The osmometer tubes were rocked

in an immersion bath kept iced at a temperature of about 8°C. After rocking in the bath for 24 hours, the osmotic pressure showed little or no change but the rocking was continued for 12 hours longer. The osmotic pressure was then read, the tubes removed from the bath, and the inside and outside fluid collected. When potential was to be read part of the inside and outside fluid was restored to smaller tubes similar to Fig. 3 of Northrop and Kunitz using about half of the same collodion sac employed for the osmotic equilibrium. The potential across the membrane was read at room temperature. Analyses on the inside and outside fluids were made by the following methods: hemoglobin was determined by method of Stadie (7); chloride by the method of Van Slyke (8); total base by the method of Stadie and Ross (9); CO<sub>2</sub> was determined by Van Slyke's method; pH was determined electrometrically on the outside solution at 38°C. Two or three osmometers were filled in each experiment and the contents combined for analysis. Any experiment in which hemoglobin staining of the outer solution occurred was discarded.

### Results.

*Hemoglobin Solution with Added Salt.*—In Table I are given the data on two experiments in which the alkaline hemoglobin solution with about 100 mM NaCl was dialyzed against a solution of 100 mM NaCl in water.

From the theoretical considerations it follows that the observed osmotic pressure must be somewhat greater than the osmotic pressure due to the hemoglobin. The observed osmotic pressure is less than half that calculated for an ideal solution whose solute concentration is the equivalent of the hemoglobin concentration expressed as 1 mol of hemoglobin per mol of oxygen capacity. At the top of Table I are given the calculated osmotic pressures of the hemoglobin according to the formula of Adair,  $\pi \text{ mm} = \frac{10.21}{4} \left( \frac{C}{1-0.015 C} \right)$ , where C = gm. of Hb per 100 cc. of solvent = 1.67 [Hb]. It will be seen that the observed osmotic pressures are in excess of the pressures calculated by Adair's formula for  $\pi_{\text{Hb}}$  by approximately the amount expected from Fig. 1.

In these experiments we confirm the observation of Van Slyke, Wu, and McLean that the distribution of base and chlorides across the membrane is approximately consistent with the Donnan equilibrium when expressed in terms of concentration per kilo of water. As pointed out by Van Slyke, Hastings, Murray, and Sendroy (10) this gives, however, only an approximation to the activity ratios.



*Hemoglobin Solution with No Added Salt.*—In accordance with theory, when no salt was added to the alkaline hemoglobin solution, the osmotic pressures observed were higher as shown in Table II. From the analysis of base outside, it is evident that considerable dialyzable anion must have been present in these

TABLE I.  
*Hemoglobin Solution with Added Salt.*

	Experiment 1.	Experiment 2.
t° of osmotic equilibrium.....	7°	7°
$\pi$ observed { mm. Hg.....	75	78
{ mm.....	4.3	4.5
$\pi_{Hb}$ calculated according to Adair { mm. Hg.....	57	67
{ mm.....	3.3	3.9
Concentrations inside per liter.		
(Hb) mm O <sub>2</sub> capacity.....	8.60	9.47
(B) <sub>i</sub> m.-eq.....	109	110
(Cl) <sub>i</sub> mm.....	84.5	84.1
Concentrations inside per kilo H <sub>2</sub> O.		
[Hb] mm O <sub>2</sub> capacity.....	10.0	11.3
[B] <sub>i</sub> m.-eq.....	126.7	131.1
[Cl] <sub>i</sub> mm.....	98.2	100.2
Concentrations outside per liter.		
(B) <sub>o</sub> m.-eq.....	118	114
(Cl) <sub>o</sub> mm.....	102.9	108.6
(CO <sub>2</sub> ) <sub>o</sub> mm.....	3.5	1.7
Concentrations outside per kilo H <sub>2</sub> O.		
[B] <sub>o</sub> m.-eq.....	118.5	114.5
[Cl] <sub>o</sub> mm.....	103.3	109.0
[CO <sub>2</sub> ] <sub>o</sub> mm.....	3.5	1.7

solutions. Only part of this was due to CO<sub>2</sub>. Qualitative tests for Cl<sup>-</sup> were negative. An unidentified anion was also present in the experiments with added salt as may be seen by noting the difference between (B)<sub>o</sub> and the sum of (Cl)<sub>o</sub> and (CO<sub>2</sub>)<sub>o</sub> in Table I. We have no analysis of the diffusible anion inside but if we assume complete dissociation of the base bound by hemo-

globin then the concentration ratios expressed per kilo of water should here as in the experiments with added salt approximately satisfy the Donnan equilibrium. It would then follow that

$$[A]_i = [A]_o \frac{[B]_o}{[B]_i} \quad (B)_o \frac{[B]_o}{[B]_i}$$

TABLE II.  
*Hemoglobin Solution without Added Salt.*

	Experiment 3.	Experiment 4.	Experiment 5.
t° of osmotic equilibrium.....	7°	8°	8°
π observed { mm. Hg.....	181	115	157
{ mm.....	10.4	6.6	9.0
π <sub>mm</sub> calculated per kilo H <sub>2</sub> O.....	27.0	18.2	23.5
"      "      "      , π <sub>Hb</sub> = 0.....	17.9	10.0	12.1
"      "      "      , π <sub>Hb</sub> calculated			
according to Adair.....	20.8	12.5	16.0
Concentration inside per liter.			
(Hb) mm O <sub>2</sub> capacity.....	7.89	7.5	9.75
(B) <sub>i</sub> m.-eq.....	23.5	12.9	23.7
Concentration inside per kilo H <sub>2</sub> O.			
[Hb] mm O <sub>2</sub> capacity.....	9.1	8.2	11.4
[B] <sub>i</sub> m.-eq.....	27.0	14.1	27.7
Concentration outside.			
(B) <sub>o</sub> m.-eq. = [B] <sub>o</sub> m.-eq.....	4.98	2.27	9.42
(CO <sub>2</sub> ) <sub>o</sub> mm = [CO <sub>2</sub> ] <sub>o</sub> mm.....			3.1
pH <sub>38°</sub> .....	7.2	7.9	7.6
t° of potential measurement.....			23°
E <sub>mem</sub> volts.....			>0.004 <0.010

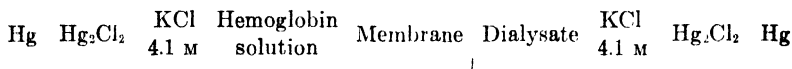
We may then calculate the expected osmotic pressure:

$$\pi_{mm} = [B]_i + [A]_i + [Hb] - 2 (B)_o.$$

We found, however, in the experiments with added salt that the osmotic effect of [Hb] was less than the expected. We have accordingly also calculated π from the above equation with π<sub>Hb</sub>

taken as zero and with  $\pi_{\text{Hb}}$  calculated according to Adair. The values for  $\pi$  thus calculated are at the top of Table II. It is evident that even if the osmotic effect of  $[\text{Hb}]$  be taken as zero the expected value of  $\pi$  on the assumption that all the base bound by hemoglobin is dissociated is much higher than the observed value. If we use Adair's values for  $\pi_{\text{Hb}}$  then the observed  $\pi$  is from 50 to 56.3 per cent of the calculated  $\pi$ . This agrees with Adair's observations. It suggests as found by Adair that the base bound by hemoglobin is not dissociated to the extent that an equivalent amount of  $\text{BCl}$  would be.

Independent evidence can be obtained regarding this if we can measure the potential across the membrane. In the last experiment this was attempted. The cell set up was



It was measured at 23°C. The results were not highly satisfactory because of the high impedance in the system. All that can be stated is that deflection of the galvanometer was definite when the cell was balanced against an E.M.F. of less than 0.004 or more than 0.010 volts. The relation between  $E_{\text{mem}}$  and activity ratios at 23°C. is

$$\frac{E_{\text{me}}}{0.0586} = \log \frac{\alpha_{\text{B}_i}}{\alpha_{\text{B}_o}} \quad \log \frac{\alpha_{\text{A}_o}}{\alpha_{\text{A}_i}}$$

Therefore for  $E_{\text{mem}} = 0.004$  and 0.010 the values of  $\frac{\alpha_{\text{B}_i}}{\alpha_{\text{B}_o}} = 1.2$  and 1.5 respectively.

If even the higher of these ratios be compared with the ratio of analyzed base per kilo of water inside and outside, namely  $\frac{[\text{B}]_i}{[\text{B}]_o} = \frac{27.7}{9.42} = 2.94$ , it becomes evident that the activity of  $[\text{B}]_i$  which consists chiefly of base bound by hemoglobin is only about half that indicated from the concentration of base per kilo of water. Therefore the evidence from the membrane potential measurements is not inconsistent with that from the osmotic pressure measurements to the effect that the activity,  $\alpha_{\text{B}_i}$  of the base in the hemoglobin solution is only about half its concen-

tration per kilo of water  $[B]_i$ . In the experiment with added salt, however, we observed that when the base and chloride concentrations in the hemoglobin solution are expressed per kilo of water the Donnan equilibrium is approximately satisfied; or in other words, that the ratio of concentrations of base per kilo of water is approximately the ratio of activities and  $\frac{\alpha_{B_i}}{\alpha_{B_o}} = \frac{[B]_i}{[B]_o}$ .

In the experiments with added salt the base bound by hemoglobin is a relatively small part of the total base. It is only in the experiments without salt where the major part of the base inside is base bound by hemoglobin that the discrepancy between

$\frac{[B]_i}{[B]_o}$  and  $\frac{\alpha_{B_i}}{\alpha_{B_o}}$  becomes evident. This would indicate that the

activity of base bound by hemoglobin is lower than the activity of the base of BCl in the presence of hemoglobin and that the base bound by hemoglobin is not dissociated to the degree of an equivalent amount of BCl. An alternative explanation would be complete dissociation of both BHb and BCl in both types of solution with an activity coefficient for base in the presence of hemoglobin which varies with the salt content of the solution, decreasing within the range of these experiments as the salt content decreases.

#### CONCLUSIONS.

On the basis of measurements of osmotic pressure in solutions of alkaline hemoglobin solution with and without added NaCl it is concluded (1) that the osmotic activity of hemoglobin under the conditions of these experiments is less than half that calculated on the basis of 1 mol of Hb per mol of  $O_2$  capacity and that it appears to agree with Adair's formula for the osmotic pressure of pure hemoglobin, and (2) either that the base bound by hemoglobin is dissociated only to about half the extent of an equivalent amount of BCl or that dissociated base in the presence of hemoglobin has an activity coefficient that decreases as the salt content decreases, confirming Adair's observation concerning the apparent dissociation of sodium hemoglobinate.

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## STUDIES ON EXPERIMENTAL RICKETS.

### XXVII. VARIATION OF VITAMIN D CONTENT OF BUTTER FAT AS A FACTOR IN THE DEVELOPMENT OF RICKETS INDUCED BY DIETS SUITABLE FOR PREPARING RATS FOR THE LINE TEST.

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(Received for publication August 30, 1926.)

In August, 1925, we discussed diets composed principally of purified foodstuffs for use with the line test for vitamin D studies.<sup>1</sup> The following diets were used:

<i>Diet 4025.</i>		<i>Diet 4026.</i>	
Wheat germ.....	5.0	Wheat germ.....	5.0
Salt mixture 37.....	5.15	Salt mixture 38.....	4.3
CaCO <sub>3</sub> .....	1.5	CaCO <sub>3</sub> .....	1.5
Gelatin.....	10.0	Casein.....	20.0
Egg albumen.....	10.0	Gelatin.....	5.0
Wheat gluten.....	12.0	Wheat gluten.....	5.0
Agar-agar.....	2.0	Agar-agar.....	2.0
Dextrin.....	49.35	Dextrin.....	52.2
Butter fat.....	5.0	Butter fat.....	5.0

We also reported the production of rickets in rats with these diets modified in that yeast replaced wheat germ as a source of vitamin B.

These diets produced an exaggerated form of rickets. Calcification of the cartilage in the tibiae of these animals was entirely wanting. Young rats weighing 40 to 45 gm. developed severe

<sup>1</sup> McCollum, E. V., Simmonds, N., Becker, J. E., and Shipley, P. G., *J. Biol. Chem.*, 1925, lxx, 97.

rickets. They were ready to use for line testing after being confined to these diets for about 25 days. When yeast was substituted for the wheat germ in Diet 4025 they were usually not ready for the test until the diet had been fed 34 to 40 days.

In using these diets in the autumn of 1925 we observed in all cases that the bones were almost normal in structure. We were mystified by this and at first were inclined to suspect that the intensity of the light in our new rat colony, which is on the eighth floor and has very large French windows, might have afforded sufficient protection to the little rats to counteract the effects of the diet. Further studies with the curtains drawn showed that this was not the case. We then investigated the butter fat employed in feeding and found that it had a greater vitamin D value than any which we had hitherto secured. The butter was made in Wisconsin. That previously employed was guaranteed by the dealer to be Elgin butter.

We next tested these same dietary formulas including but 1 per cent and 2 per cent respectively of butter fat in different experiments. In these cases we observed very severe rickets in all animals. Those receiving 2 per cent of butter fat were in a little better condition than those which had but 1 per cent.

When using diets such as those here described for testing natural foods for vitamin D, it is essential to keep in mind that the phosphorus and calcium content must be kept constant throughout the series of modified diets. Raising the phosphorus content of the food tends to enable the animals to deposit calcium phosphate in the bones. Regulation of the phosphorus content can be accomplished by replacing a part of either the casein, gelatin, or egg albumen and a part of the dextrin if necessary by such an amount of the food to be studied as will equal these in protein and phosphorus content.

Since we have chanced to use a sample of butter fat which contained more than the usual amount of vitamin D, we publish this brief account of our experience in order to point out the necessity of keeping the butter fat content of the diet as low as possible, since this may in certain cases be a disturbing factor.

## THE CHEMICAL STUDY OF BACTERIA.

### XII. THE ALBUMIN-GLOBULIN FRACTION OF THE TUBERCLE BACILLUS.\*

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(Received for publication. June 28, 1926.)

#### INTRODUCTION.

This paper, and one to follow, are preliminary reports dealing with the preparation, methods of purification, and proximate analysis of certain specific proteins present in the tubercle bacillus. Due to the great complexity of an aqueous extract of any complete organism, such as a bacterium, it is very difficult to separate a given product from such solutions in the state of purity which is to be desired. This is particularly true of the protein constituents of cells which, in addition to being separated from one another, must also be freed from adsorbed impurities. Many experimental difficulties are encountered in the development of a technique which can be recommended for general application in chemical work of this character, and until much larger quantities of bacteria are available, the further purification of these protein fractions must be postponed. In the light, however, of important results which have been reported by other workers in the biological field, and whose researches are dependent on the progress and success of our work, it has become particularly advisable to publish certain findings which have been made to date.

In a previous publication (1) has been charted the procedure which has so far been developed for the separation of the various constituents of the bacterial cell. The present work has been

\* This work is part of a cooperative research carried on under the auspices of the Research Committee of the National Tuberculosis Association, and was supported by a special grant from that committee.

† National Tuberculosis Association Research Fellow.



carried out in accordance with that plan except for several minor operations which are now discussed in greater detail. All of the bacilli used were grown by the H. K. Mulford Company of Philadelphia. The medium used was made entirely from chemicals of established purity. This eliminates the possibility that any product obtained from the cells was not synthesized by the bacteria. The bacteria used were a human strain, H37. The culture media had the composition shown in Table I.

After 6 to 8 weeks growth under sterile conditions, the bacteria were filtered off on Buchner funnels, washed free from chlorides with distilled water, and dried in a vacuum oven at a temperature which was never greater than 50°C. They were then ground in a mortar or porcelain ball mill, and the resulting nearly white powder

TABLE I.  
*Composition of Medium.*

	<i>gm.</i>
Glycerol.....	50.0
Asparagine.....	5.0
Sodium citrate.....	6.0
KH <sub>2</sub> PO <sub>4</sub> .....	2.0
NH <sub>4</sub> Cl.....	1.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5
Ferrous ammonium citrate.....	0.05
Water.....	1000

dried to constant weight in a vacuum desiccator over sulfuric acid. After separation from the culture media, it is necessary that the bacteria be thoroughly washed with water and filtered as dry as possible before being placed in the vacuum drying oven, otherwise a clean white powder will not be obtained after grinding.

#### EXPERIMENTAL.

Before the bacteria are treated to remove their water-soluble constituents it is necessary to extract them with an organic solvent to remove fat. Although the ordinary bacteria contain only about 2 to 5 per cent of fat, the bacteria of the acid-fast group, to which the tubercle bacillus belongs, are characterized by their high lipid content, running from 15 to 50 per cent of the weight of

the desiccated cells. This fat encases the bacteria and cements them together and must be removed in order to get at the interior of the cell.

To avoid any changes which heat might induce in the protein constituents, this defatting process was done at room temperature. Experience with different procedures showed that decantation with cold ether was the best method. Four such treatments, allowing the ether to stand on the bacteria for 2 or 3 days each time, give practically a complete extraction, the fourth extract being nearly fat-free. This treatment does not remove all the fat and waxes of the bacteria, but merely that portion which constitutes the protective coating of the cells and is easily soluble in ether. Unless this is first removed it is not possible to accomplish a quick extraction of the protein fraction with cold water. With the bacteria used in this research, the yield of fat varied from 18 to 20 per cent of the weight of the dried cells.

The subsequent treatment to obtain the water-soluble proteins will be discussed under two sections. In the first treatment distilled water was used immediately after the defatting of the bacteria, and in the second, the initial solvent used was a 5 per cent aqueous solution of sodium chloride.

#### *A. Water as Initial Solvent.*

100 gm. of dry bacteria were defatted as described above, 20.3 per cent of fat being obtained. Three extractions with cold distilled water were then made, the bacteria being allowed to macerate for 36 hours each time. Toluene was used as a preservative. These extracts, made at room temperature, were then combined to give a total volume of 2380 cc. This solution was centrifugalized as well as possible in a cup centrifuge rotating at a speed of 1500 revolutions per minute, and the protein precipitated by addition of acetic acid.

The protein thus obtained was separated by means of the centrifuge and redissolved in the least possible amount of 1 per cent sodium hydroxide solution. A small quantity of insoluble residue was removed by centrifugalizing and the protein reprecipitated with acetic acid and again submitted to this same process of purification. After the third precipitation with acetic acid, the protein was washed once with 2 per cent acetic acid, twice with 95

per cent alcohol, twice with ether, and then dried in a vacuum desiccator over sulfuric acid. The yield was 6.0 gm. of a nearly white powder.

This protein<sup>1</sup> gives negative tests for sugar with both Fehling's and Benedict's solutions, but a very strong Molisch test. It also gives all the ordinary protein reactions. The results of analyses and nitrogen distribution are recorded in Table II. The sulfur content of the basic fraction of this protein was so low that it could not be satisfactorily determined. The calculations for nitrogen

TABLE II.  
*Analysis of Water-Soluble Protein.*

	Per cent.	Per cent of total N.
Nitrogen (Kjeldahl) . . . . .	12.6	100
Tryptophane (May and Rose (2)) . . . . .	1.5	
Nitrogen distribution (Van Slyke).		
Humin . . . . .		4.4
Amide . . . . .		9.0
Phosphotungstic filtrate . . . . .		46.2
Amino . . . . .		39.8
Non-amino . . . . .		6.4
Bases . . . . .		37.1
Arginine . . . . .	8.7	22.2
Histidine . . . . .	4.7	10.1
Lysine . . . . .	3.1	4.8
Total . . . . .	18.0	96.7

distribution were made assuming the absence of cystine, although there is possibly a trace present.

A second experiment was run with a few variations in procedure. In this case 340 gm. of bacteria were taken. Extraction of the cells with ether removed 18.4 per cent of fat. The bacteria residue was extracted with distilled water at room temperature for 2 days and then centrifugalized as well as possible in a cup centrifuge at a maximum speed of 1500 R.P.M. During this time chloroform

<sup>1</sup> No. 502 according to our laboratory record. This fraction, corresponding to section (S) of our published scheme of analysis (1), has been reported to us as being a very strong tuberculin by Dr. Esmond R. Long of the Sprague Institute, University of Chicago (unpublished).

was used as a preservative. This brings it to the stage where in the above experiment the protein was precipitated with acetic acid. In this experiment, however, the water extract was twice run through a Sharples supercentrifuge at a speed of 45,000 R.P.M. In this manner we succeeded in obtaining a clear and light brown fluorescent liquid. Acetic acid was added to this to maximum precipitation, and without further purification, the protein which separated was washed with dilute acetic acid, 95 per cent alcohol, and ether, and finally dried in a vacuum desiccator over sulfuric acid. The desiccated protein weighed 3.03 gm. and contained 15.5 per cent of nitrogen. It gave the usual protein reactions, including the Molisch test, but it responded to no other tests for sugar either before or after hydrolysis.

The bacteria residue from the first aqueous extraction was given a second and third extraction with water in just the same manner

TABLE III.  
*Partition of Extractable Protein.*

Extraction. ....	First.	Second.	Third.	Total.
Weight of protein, gm. ....	3.03	0 35	None.	3 38
Nitrogen in protein, per cent....	15.5	11.1		

as the first. The results of these extractions are summarized in Table III

#### *B. 5 Per Cent Sodium Chloride as Initial Solvent.*

100 gm. of bacteria were defatted by maceration and decantation with ether, yielding 18.4 gm. of fat. They were then treated with 600 cc. of a 5 per cent sodium chloride solution and shaken occasionally over a period of 2 weeks, chloroform being used to maintain a sterile condition. This caused them to swell to ten times their original volume. The supernatant extract was then carefully decanted and the residue washed four times by decantation with 200 cc. portions of 5 per cent sodium chloride solution.

The brine extract and washings were combined and then centrifugalized in the cup centrifuge at a speed of 1500 R.P.M. The solution, which was now slightly fluorescent and of a yellowish brown color, was twice run through the Sharples supercentrifuge

at a speed of 35,000 R.P.M. It came out sparkling clear and showed an acidity corresponding to pH 6.0. This solution was then dialyzed free from chlorides in a parchment sac in the presence of toluene. This caused the separation of a minute amount of a colorless flocculent precipitate, which, however, gave a negative biuret test, showing its non-protein character. Judging from this result we conclude that the tubercle bacillus must contain a very small amount of a globulin, if any.

After dialysis, the aqueous solution was saturated with ammonium sulfate and the precipitated protein filtered off, dissolved in fresh water, and again dialyzed under aseptic conditions until Nessler's solution gave only a slight test for ammonia and the test for sulfate radical was entirely negative. Two volumes of

TABLE IV.  
*Summary of 5 Per Cent Aqueous Sodium Chloride Extracts.*

	Experiment I.			Experiment II.		
	<i>gm.</i>	<i>per cent</i>	<i>per cent of total N</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent of total N</i>
Bacteria .....	100	8.68	100	100	8.47	100
Fat removed .....	18.4	18.4		20.6	20.6	
N in defatted bacteria.....		10.6			10.7	
“ “ NaCl extract.....	3.075		35.5	3.049		36.0
Globulin .....	None.			None.		
Water-soluble protein.....	0.50	11.3	0.65	0.67	9.35	0.74

95 per cent alcohol were then added and the resulting protein precipitate was washed twice each with 95 per cent alcohol and ether and finally dried in a vacuum desiccator over sulfuric acid. As it dried black, it was redissolved in water, precipitated with alcohol again, and dried by washing twice with 95 per cent alcohol, once with absolute alcohol, twice with absolute ether, and once with dry toluene. In applying these washings use was made of the centrifuge as efficient filtering was impossible.

This protein, after being dried in a vacuum desiccator, was obtained as a light gray powder which contained 11.3 per cent nitrogen. It gave all the usual protein tests, including a strong Molisch reaction, but did not contain enough potential reducing substances to give *even a trace* of reduction with Benedict's solution after hydrolysis.

A second experiment was run in exactly the same manner, to serve as a check on the first. In this case no precipitate was obtained upon dialysis of the original extract, which corroborates the non-protein character of the precipitate obtained at this point in the first experiment. The results of these two experiments are conveniently summarized in Table IV.

#### DISCUSSION.

A careful analysis of the analytical data recorded in Table IV reveals some very important facts which will have an immediate influence on the future course of our researches on tubercle bacilli. As mentioned in the first part of this paper, it must be borne in mind that the water extract of bacteria is a very complicated mixture. Table IV shows that extraction with cold 5 per cent sodium chloride solution removes 36 per cent of all the nitrogen of the bacteria. In Experiment I, after dialysis only 0.655 gm. of nitrogen remained within the parchment sac, showing that at least 2.42 gm., or 28 per cent of all the nitrogen in the tubercle bacilli, is in the form of non-protein, dialyzable compounds. In what organic combinations it exists is a problem which must be decided by future research. To date the only definite nitrogen-containing constituent that has been separated from it is a small amount of free tyrosine, and from this result it might be expected that other amino acids are floating free within the bacterial cell. Whether this aqueous extract contains sugar in other combinations than are found in nucleic acids remains to be determined.

Another interesting point to be emphasized is in connection with the persistent Molisch test which is encountered in all the preparations. This test is so delicate that it will serve to detect the smallest traces of a carbohydrate grouping, so it is not surprising that proteins prepared from a bacterial extract should give the reaction. A fresh extract of the tubercle bacillus will not reduce Fehling's or Benedict's solutions until *after* acid hydrolysis, when it gives a very strong reduction. This is due to the presence of glycogen and nucleic acid existing in the free condition (3), which breaks down on hydrolysis, and also to the polysaccharide combination which Mueller (4) and Laidlaw and Dudley (5) have succeeded in isolating, and which is the specific substance in the precipitin reaction. Thus in the water extract of this bacteria

we are dealing with proteins, nucleic acid, glycogen, a polysaccharide, free amino acids, inorganic salts, and probably several other organic compounds as yet unknown.

An interesting difference is noted in the nitrogen content of the protein fractions prepared by acetic acid precipitation of a water extract. In the first case, where the extract was centrifugalized at a speed of only 1500 R.P.M. the protein had a nitrogen content of 12.3 per cent. In the next experiment, the Sharples supercentrifuge was used, and by means of this high speed a jelly-like substance is removed having a nitrogen content varying from 5 to 9 per cent of this element in different experiments. As a result of removing this substance, the yield of the protein fraction is reduced from 6 to 1 per cent, but the nitrogen content jumps from 12.3 to 15.5 per cent. The protein with low nitrogen percentage and, we assume, of very high impurity, will act satisfactorily as an antigen in complement fixation reactions, while the product with the high nitrogen, which indicates a higher state of purity of the protein, is inactive when used biologically in the same manner (6). What this protein impurity is remains to be established by further work.

Although the literature on the proteins of the tubercle bacillus is quite extensive, only a brief mention will be made of it here. The emphasis in this work was placed on obtaining from the cell a protein in its original form if possible. To accomplish this end the bacteria were never heated, nor treated with mineral acids at all, or acetic acid above 2 per cent strength. No alkali was used in their preparation, except in one instance. The only reagents which have been used in any manner, except the one case of dilute alkali, were dilute acetic acid and the inert solvents ether, alcohol, chloroform, and toluene. On the contrary, the literature deals with proteins which have been obtained by autoclaving, acid and alkali extractions, and other means which would lead to deep seated changes in the protein molecule. The products obtained hitherto have lost the structural characteristics of the proteins existing in the cell.

Due to the lack of materials, no quantitative analyses were made for sulfur or phosphorus, although qualitative tests show these elements to be present in very slight amounts.

In regard to the amino acid analyses, the high percentage of the

hexone bases is the most striking characteristic. The arginine, in particular, is much higher than is generally found in proteins with the exception of the protamines and so called histones.

#### SUMMARY.

1. A water-soluble protein having the properties of an albumin has been isolated in an unaltered condition from the tubercle bacillus.

2. Evidence is presented which would indicate that the globulin content of the tubercle bacillus is very small if any.

3. This water-soluble protein shows an unusually high content of basic amino acids.

4. The protein preparations described have been reported as having a pronounced biological activity, and further investigation dealing with their specific properties is now in progress.

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## THE CHEMICAL STUDY OF BACTERIA.

### XIII. THE ALKALI-SOLUBLE PROTEIN OF THE TUBERCLE BACILLUS.\*

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(Received for publication, June 28, 1926.)

#### INTRODUCTION.

In a previous paper (1) from this laboratory has been given the method of preparation and analysis of the water-soluble protein of the unautoclaved tubercle bacillus. This paper will deal with that protein which is obtained by maceration, with dilute alkali, of the cell residue left after complete extraction of the defatted bacteria with cold water. This work, as in the case of the work discussed in the preceding article, was carried out in the main part according to the plan outlined in a previous paper (2) and concerns itself with the protein fraction marked O as indicated in the chart in that publication.

The material used in this research was the bacterial residue which was left after the cold water extractions described by the writer in the previous paper (1) of this series. The original material was a quantity of unautoclaved human tubercle bacilli grown on synthetic media and treated, thereafter, in such a manner as to cause the minimum chemical change in the protein fractions of the organism.

#### EXPERIMENTAL.

The two experiments to be described in this paper were carried out with the bacterial fractions left behind after extraction of two

\* This work is part of a cooperative research being carried on under the auspices of the Research Committee of the National Tuberculosis Association, and was supported by a grant from that committee.

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units of tubercle bacilli with sodium chloride solution. The preliminary operations have already been discussed in Part B of the preceding paper (1). Although several other experiments have been run through, these two are representative and will serve to illustrate our procedure of analysis of this fraction of the cell. After the extractions with 5 per cent sodium chloride solution, the bacteria were extracted once with cold water to remove the most of the remaining salt solution. The extract was then run through a Sharples supercentrifuge to remove all solid particles, and the nitrogen content of the resulting clear solution determined. This proved to be relatively small in each case and was discarded. After weighing this bacterial residue it was possible to calculate, with sufficient accuracy, the amount of water present. To this was then added an aqueous solution of sodium hydroxide of such a strength that it made a total of 1 liter of 0.5 per cent alkali. This mixture was then allowed to macerate a week at ordinary temperature with occasional shakings. The mixture was kept sterile throughout this operation. By the end of this time the odor of ammonia was quite evident, and the bacterial residue had become noticeably darker in color. The consistency had also changed. Whereas, before extraction it had been in distinct clumps very much resembling macerated bread, it was now homogeneous, or apparently so, throughout. Nothing would settle out on standing or upon centrifugalizing in a fast cup centrifuge. Upon running it through the supercentrifuge, however, a large amount of a jelly-like substance was removed, which not only gave the customary protein reactions, but also gave very strong tests for reducing sugars *after* acid hydrolysis. It was necessary to centrifugalize this alkaline solution repeatedly before it became clear. At this point acetic acid was added to maximum precipitation and the protein obtained by removing it with a centrifuge and washing twice each with 95 per cent alcohol and ether, the washings also being made by means of the centrifuge.

The protein thus obtained was a nearly colorless powder, which was strongly electrified. It was insoluble in water but dissolved readily in very dilute alkali or in buffer solutions having a pH of 7.6 or greater. It gave all the usual protein reactions, the Moliach test being weak in comparison with that given by the water-

soluble protein described in our previous paper (1). No other sugar tests could be obtained either before or after hydrolysis of the protein. Although impurities of a carbohydrate nature were very slight, as shown by the Molisch test, lipids or waxy products were present in considerable amount. When 1 gm. of the protein was decomposed with aqua regia in order to make a phosphorus determination, a waxy substance was liberated which withstood 4 hours boiling with several portions of aqua regia. It was readily soluble in toluene, and on evaporation of the solvent and drying

TABLE I.  
*Distribution of Nitrogen in Tubercle Bacillus.*

	Experi- ment I.	Experi- ment II.	Experi- ment I.	Experi- ment II.	Experi- ment I.	Experi- ment II.
	gm.	gm.	per cent N	per cent N	per cent of total N	per cent of total N
Bacteria . . . . .	100	100	8.68	8.47	100	100
Fat content . . . . .	18.4	20.6				
N in 5 per cent NaCl extract . .	3.075	3.049			35.5	36.0
Water-soluble protein . . . . .	0.50	0.67	11.3	9.35	0.65	0.74
N in water extract . . . . .	0.693	0.231			8.0	2.7
" " 1st extract alkali . . . . .	*	3.792			*	44.7
NaOH-soluble protein . . . . .	10.8*	20.4	14.2	13.8	17.7*	33.2
N in protein filtrate . . . . .	0.512*	0.518			5.9*	6.1
N in 2nd alkali extract . . . . .	0.691*	0.321			8.0*	3.8
Protein . . . . .	3.3*	1.7	11.7	11.6	4.5*	2.3

\* While running the first alkali extract through the centrifuge, the machine ran off center and a part of the extract, with its suspended solids, was unavoidably lost.

in the air had a weight of 0.325 gm. The presence of this characteristic material in the alkali-soluble protein has also been noticed in other preparations, even those which were submitted to a purifying process of three successive solutions in dilute alkali with subsequent reprecipitation of the soluble protein.

The residue from the first alkali extraction was again extracted with the same solvent, and the extract treated in exactly the same manner as described above.

In Table I the results of two parallel experiments are so recorded as to show the distribution of nitrogen in both the water- and

alkali-soluble portions of the cell. The figures for the water-soluble portion are taken from the preceding paper (1).

The proteins prepared in both experiments were analyzed

TABLE II.  
*Van Slyke Analysis of Alkali-Soluble Protein.*

	Experiment I.	Experiment II.
	<i>per cent of total N</i>	<i>per cent of total N</i>
Humins .....	2.5	3.4
Amides .....	4.7	3.9
Bases .....	29.3	29.5
Arginine .....	17.3	17.8
Histidine .....	5.0	4.2
Lysine .....	7.0	7.5
Phosphotungstic acid filtrate .....	62.3	63.6
Amino .....	57.6	62.6
Non-amino .....	4.7	1.0
Total .....	98.8	100.4

TABLE III.  
*Analyses of the Alkali-Soluble Protein.*

	<i>per cent</i>
Nitrogen (Kjeldahl) .....	13.8
Phosphorus (3) .....	0.9 (as $P_2O_5$ )
Sulfur (3) .....	0.65
Cystine (4) .....	0.9
Tryptophane (5) .....	1.9
Tyrosine (6) .....	0.9
Arginine (Van Slyke) .....	7.6
Lysine ( " " ) .....	5.5
Histidine ( " " ) .....	2.1
" (6) .....	2.7
Total of amino acids .....	18.2 (Using the average of the two histidine values.)

according to the Van Slyke method for determining the nitrogen distribution. In addition, the protein obtained in Experiment II was analyzed for sulfur, phosphorus, cystine, tryptophane, tyrosine, and histidine. Table II gives the results of the Van

Slyke analysis of the two proteins. The sulfur in the basic fraction was too small in amount to determine successfully, so the calculations were made assuming the absence of cystine, although there is probably a small amount present.

In Table III are given the analytical values other than the Van Slyke, and the latter are changed from per cent of the total nitrogen to per cent of the amino acid in the protein.

#### DISCUSSION.

It is evident from an examination of Table I, that the bulk of the protein content of the tubercle bacillus is in the alkali-soluble portion of the cell. Approximately 20 per cent of the weight of the bacteria and 33 per cent of all the nitrogen occur in this form. This amount is in marked contrast to the small amount, 1 per cent of the bacteria, which occurs as a water-soluble protein.

That the water-soluble protein is not the same as the alkali-soluble product is amply proven by the analytical results obtained by the Van Slyke procedure. The water-soluble protein has the higher content of bases, 37.1 against 29.4 per cent in the alkali-soluble protein. This is a contributing factor to even a greater difference in the nitrogen content of the filtrate from the phosphotungstic acid precipitation of the bases. In the water-soluble protein this fraction contains only 40 per cent of the nitrogen, against the much higher value of 63 per cent in the alkali-soluble product. Among the bases themselves there are also notable differences. The arginine and histidine are higher in the water-soluble protein, while the lysine is low in this fraction and high in the alkali-soluble form.

The amide figures must not be taken at present as indicating anything very definite. As stated above, the odor of ammonia was quite pronounced while the alkali extraction was being made. Whether this represented the amide nitrogen of the protein is a question. It seems quite probable that it was, at least in part. This would help, therefore, to account for the low amide nitrogen content of the alkali-soluble protein. However, there is another consideration which cannot be overlooked in this connection. According to present theories of protein structure (7), the amide nitrogen content of a protein is practically equivalent to the number of dibasic acid molecules. It is thought to exist in the

form of an amide, neutralizing the second carboxyl of the acid. According to Campbell (8), a sample of fat-free tubercle bacilli analyzed by him contained 3.4 per cent of glutaminic acid, which would indicate that the protein contains enough dibasic acids to account for several per cent of amide nitrogen.

The occurrence of cystine in the tubercle bacillus is a question which has not yet been settled. The colorimetric method of Folin and Looney shows that there is present 0.9 per cent of this sulfur combination or an equivalent amount of a substance giving the same color reaction as cystine. Phosphotungstic acid does not precipitate sulfur in an amount to account for 0.9 per cent of cystine, although the sulfur content of the protein is twice enough to account for it.

The phosphorus content of the protein is quite significant. While the quantity found allows for 4 per cent of nucleic acid as a maximum, it is not believed that our product is a representative of the nucleoproteins. All the tests for sugar, with the exception of the weak Molisch test, were negative, and, in addition, no color test for guanine could be obtained, although it could be detected when 4 per cent of yeast nucleic acid was added to it. It may be that the phosphorus and the lipid mentioned in the experimental part of the paper are both combined with the protein in something in the nature of a lipoprotein. Long (9) has discussed this from the point of view of acid fastness, and proposes the theory that the fat is emulsified within the protein portion of the cell, and that the lipid-protein union is physical, not chemical.

It is interesting to note that in contrast to the water-soluble protein, which was an exceedingly active tuberculin, the alkali-soluble protein showed only a very low potency when tested with guinea pigs (10). This may be due to the fact that it is a non-specific protein carrying a slight amount of the water-soluble form as an impurity, or that its specificity has been destroyed by treatment with 0.5 per cent alkali.

#### SUMMARY.

1. After removal of the water-soluble constituents of the un-autoclaved tubercle bacillus, an alkali-soluble protein has been obtained, containing 14.2 per cent of nitrogen, and in an amount equivalent to 20 per cent of the weight of the desiccated bacterial cell.

2. This protein is markedly different chemically from that protein which is extracted with water, being less basic in character.

3. The alkali-soluble protein, in contrast to the water-soluble protein, is practically devoid of tuberculin action.

In conclusion, I wish to thank Dr. D. Breese Jones, of the Protein Laboratory, Bureau of Chemistry, Washington, D. C., for his kindness in analyzing the protein for cystine and tryptophane, and Drs. Long and Seibert of the University of Chicago for their assistance in making the biological tests.

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# ON THE PRESENCE OF AMINES IN THE DISTILLATE FROM KJELDAHL-GUNNING NITROGEN DETERMINATIONS.

## PRELIMINARY PAPER.\*

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(Received for publication, August 20, 1926.)

We have recently been conducting an extensive study of the effect of various inorganic salts on the extraction of proteins from wheat flours. The amount of protein extracted was followed by making Kjeldahl nitrogen determinations on aliquots of the extract. When certain salts were used, the odor of (presumably) methyl amines became so pronounced in the distillates when they were made alkaline as to suggest that a considerable part of the nitrogen was being distilled over in the form of amines rather than as ammonia. A search of the literature has so far failed to reveal any definite proof that the Kjeldahl-Gunning process yields only ammonia, for the titration of the amines will, of course, indicate the same nitrogen percentage as would be found if all of the nitrogen were in the form of ammonia.

We have accordingly attempted to determine whether or not aliphatic amines are actually present in the distillates from normal Kjeldahl-Gunning digests and what effect the presence of added salts may have upon their presence. Due to the fact that one of us (W.F.H.) can no longer work upon this problem, it has seemed wise to present this preliminary report in spite of the fact that no study has as yet been made as to the chemical identity of the amines which are present.

The method of Weber and Wilson<sup>1,2</sup> was used to estimate the

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<sup>1</sup> Weber, F. C., and Wilson, J. B., *J. Biol. Chem.*, 1918, xxxv, 385.

<sup>2</sup> Weber, F. C., and Wilson, J. B., *J. Ind. and Eng. Chem.*, 1919, xi, 121.

amines in the presence of ammonia and has proven very satisfactory.

In our Kjeldahl determinations we have used 25 cc. of nitrogen-free concentrated  $\text{H}_2\text{SO}_4$ , 10 gm. of potassium sulfate, and about 1 cm. of No. 20 copper wire as a catalyst. The digestion was continued for about 20 minutes after the solution became colorless. No effort was made to keep the time of digestion the same, which possibly may account for the differences noted in the percentage of amine nitrogen in the distillates of duplicate samples.

It is evident from Table I that a part of the nitrogen in a Kjel-

TABLE I.

*Showing the Presence of Amines in a Normal Kjeldahl-Gunning Distillate and the Increase in Amines Due to the Presence of Magnesium Sulfate. 0.2000 Gm. of Casein Used in Each Determination.*

Added salt.	Total nitrogen.	Amine nitrogen.	
		In distillate.	In total nitrogen.
	mg.	mg.	per cent
Normal digest . . . . .	28.25	1.96	6.93
" " . . . . .	28.23	1.96	6.94
" " . . . . .	28.07	2.14	7.62
1 gm. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	28.16	2.60	9.23
1 " " . . . . .	28.14	2.56	9.10
1 " " . . . . .	28.20	2.40	8.51
5 " " . . . . .	28.30	4.28	15.12
5 " " . . . . .	28.30	5.44	19.22
5 " " . . . . .	28.17	5.32	18.88

dahl-Gunning distillate is present in the form of amines, and that the presence of magnesium sulfate greatly increases the proportion of the amines. In the case of wheat flour samples digested in the presence of 10 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  without potassium sulfate 12.5, 14.3, and 14.7 per cent of the total nitrogen in the distillate was present as amines. Qualitative tests show that when calcium chloride, strontium chloride, or barium chloride are added to a Kjeldahl-Gunning digest, they strongly influence the formation of amines. It is planned to make an extended study of this phenomenon.

## SUMMARY.

1. Amines are present in the distillate from a normal Kjeldahl-Gunning digest to the extent of approximately 7 per cent of the nitrogen.

2. Salts of the dibasic metals, Mg, Ca, Sr, and Ba markedly influence the amount of amines which are present in such a distillate. Nearly one-fifth of the total nitrogen may be in the form of amines, if the digest is made in the presence of 5 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .



## DETOXICATION OF AROMATIC CYANIDES.

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(Received for publication, June 1, 1926.)

The toxicity, physiologic effects, and antidotes are the points emphasized in the existing literature on the cyanides; Giacosa and Baumann alone have been interested in their fate in the body. Reports are unanimous that the aliphatic cyanides split off hydrocyanic acid, and that toxicity is directly proportional to the rate at which this splitting occurs, while an increase in the excretion of thiocyanates and urea has been observed after ingestion. Of the aromatic series benzaldehyde cyanhydrin, benzyl cyanide, phenyl cyanide, toluyll cyanide, *p*-hydroxybenzyl cyanide, and diethylaminobenzyl cyanide have been studied.

Reid Hunt (1) found that the molecule of benzaldehyde cyanhydrin is isotoxic with the molecule of hydrocyanic acid for mice; Wirth (2) made the same observation on rabbits. Meurice (3) did not find the two molecules isotoxic. Sulfur compounds, such as sodium thiosulfate, thialdin, copper sulfate, and ferrous sulfate, are effective antidotes for the former. Meurice found that cobalt nitrate and nickel nitrate detoxicate it in the rabbit, but not in the pigeon or dog.

Giacosa (4) reports that benzyl cyanide is extremely toxic for young animals, but that full grown animals can withstand fairly large doses. Dog urine gave a Millon reaction and contained increased quantities of ethereal sulfates. He ascribes these metabolic changes to a derangement of the organism under the influence of the cyanides. After feeding the pure substance, he isolated from the urine of the animal a small quantity of a crystalline product which he reported as phenaceturic acid. When phenyl cyanide was fed, some was excreted unchanged in the breath, the feces, and the urine, and there was a marked increase in ethereal sulfates. He concluded that the cyanogen radical does not break off, but that an oxidation of the benzene ring in the ortho and para position occurs.

Baumann (5) repeated Giacosa's experiments. On one occasion he fed 25 gm. of phenyl cyanide to a dog in the course of 3 days; he extracted the urine with ether and hydrolyzed the impure extract with strong hydro-

chloric acid, and obtained 0.27 gm. of *p*-hydroxybenzoic acid and 0.114 gm. of pure salicylic acid.

Czapek, Verbrugge, and Meurice worked with toluyl cyanide. Verbrugge states that simultaneous administration of toluyl cyanide and sodium thiosulfate does not lessen the toxicity of the former for rabbits (6) and Meurice reports the same thing in the case of the pigeon. Verbrugge found 1 gm. of the para compound fatal when injected subcutaneously into a rabbit; the toxicity of the ortho compound is about one-third less. Poisoning by toluyl cyanide does not give the symptoms characteristic of hydrocyanic acid poisoning.

Reid Hunt (1) shows that the fatal dose of *p*-hydroxybenzyl cyanide for the mouse lies between 0.07 and 0.11 mg. per gm. of body weight. He finds that the molecule of diethylaminobenzyl cyanide is slightly more toxic than that of hydrocyanic acid. Sodium thiosulfate protects against 1.4 times the fatal dose of the poison and thialdin against 1.6 times the fatal dose.

The object of our experimental work was to determine the fate of aromatic cyanides in the animal body and if possible their effect on exogenous and endogenous metabolism. Two purely aromatic cyanides, phenyl and benzyl cyanides, were chosen as types; and three substituted phenyl cyanides and three substituted benzyl cyanides were also investigated. Three obvious paths of detoxication are open to these compounds—semihydrolysis to the corresponding amide, complete hydrolysis to the corresponding acid, and oxidation of the benzene nucleus in the para position. The dog was chosen as subject. The cyanides were introduced into the stomach of the animal as a water solution by means of a stomach tube, or were injected as a solution in glycerol or olive oil. In either case there was always considerable loss of material through regurgitation or vomiting from the stomach, or from evaporation and secretion through the skin. The urine was collected periodically, but not by catheterization, for 36 or 48 hours after the last dose. The substance was usually injected in increasing quantities until at least from 3 to 5 gm. had been given.

In order to determine the effect of the nitriles on metabolism, the rabbit was chosen as the experimental animal, since on a monotonous diet of carrots, the nitrogenous output can be reduced to such a low figure that a few mg. resulting from the destruction of the cyanide would make an appreciable increase in the total nitrogen of the urinary output. The urine from the rabbit was collected in 48 hour periods and the following nitrogen determinations were made: total, urea, ammonia, and, at times, uric acid.

Inorganic, ethereal, and total sulfates, also total and reduced sulfur were checked. Specific gravity, color, odor, and reaction to litmus were noted; Millon's, albumin, and the ferric chloride test were always run, also a quantitative determination of potassium thiocyanate.

*p*-Chlorobenzyl Cyanide.

1 gm. of this substance was dissolved in olive oil and injected subcutaneously into a dog weighing 31 kilos. The dog showed no toxic symptoms. The urine was concentrated to a thick syrup, acidified, and extracted with ether. The ether extract was allowed to evaporate gradually at room temperature, but no crystals appeared. The residue was then dried over calcium chloride. It was a light brownish substance with a strong aromatic odor; it was dissolved in hot water and allowed to crystallize; on cooling 0.8 gm. of a substance was obtained which proved to be *p*-chlorobenzoic acid, as it melted at 230–231° after recrystallization and drying. After the extraction with ether, the urine was again extracted with ethyl acetate for several hours, but nothing appeared. The recovery of a glycocoll conjugate was expected, and in an effort to explain its absence, 9 gm. of pure *p*-chlorobenzoic acid were fed in 3 gm. doses to the same dog. Ether extraction of the evaporated urine gave a 10 per cent yield of the original substance fed; a subsequent ethyl acetate extraction gave 65 per cent of *p*-chlorohippuric acid, corresponding in melting point and analysis to the compound previously synthesized in this laboratory (7).

In order to trace the cyanogen group, or at least to follow up its nitrogen, a very small dog was injected with *p*-chlorobenzyl cyanide. The urine was analyzed for total nitrogen, urea nitrogen, and ammonia nitrogen through a feeding period of 6 days and for 5 days afterwards. During this period there was a decided increase in total nitrogen, with a corresponding increase in urea output, but no noticeable change in the quantity of ammonia excreted.

0.5 gm. of *p*-chlorobenzyl cyanide dissolved in olive oil was injected subcutaneously into a rabbit. Shortly afterwards the most marked symptoms of cyanide poisoning appeared; gradually respiration and pulse became slower and within 3 hours the animal died.



In another experiment, a rabbit was kept on a carrot diet and the urine analyzed for a period of 5 days for urea nitrogen, total nitrogen, and ammonia nitrogen. In addition to these, total sulfur, reduced sulfur, and ethereal sulfates were determined, also thiocyanate, which was figured as potassium thiocyanate. On the 6th day 0.25 gm. of the substance dissolved in olive oil was injected subcutaneously into the rabbit, and the dose was doubled on the following day; the rabbit died as a result of the second dose. There was very little alteration in the nitrogenous constituents of the urine, and very little in the reduced sulfur fraction, thus indicating but a slight increase in the endogenous catabolism. There was, however, a very small rise in the output of ethereal sulfates, and a decided increase in the amount of thiocyanate excreted—so much so that the 24 hour output, calculated as potassium thiocyanate, rose from an average of 2 mg. before the feeding to 11.6 mg. after the first dose, remaining at this level until death. Death was apparently caused by a gradual paralysis of the respiratory muscles.

Another rabbit was injected with 0.1, 0.2, 0.2, and 0.3 gm. of *p*-chlorobenzyl cyanide on 4 successive days. The potassium thiocyanate increased from 4.2 mg. before the feeding to 11.7, 12.3, and 14.3 mg. after the feeding, and remained at this high level for 7 days after the last feeding.

#### *p*-Nitrobenzyl Cyanide.

On account of the insolubility of this substance, the feeding of it was almost impossible; and injection was not feasible since it is insoluble in the various oils. Finally capsules coated with salol and small enough to force down a rabbit's throat proved partially successful. For the dog, similar capsules, holding 0.7 gm. of the material, were wrapped in chunks of meat.

On each of 5 successive days, a dog was fed three of these capsules, a total of 10.5 gm. Very little of the substance was absorbed, most of it passing directly into the feces without undergoing any apparent chemical change. It was found that 0.000001 of a gm. of the cyanide gave a marked pinkish coloration when made alkaline with sodium hydroxide. In the entire urine volume in this experiment, this test indicated the presence of about 0.6 gm. of the unchanged substance during the feeding period.

No trace of the unchanged material could be detected in the urine longer than 72 hours after the last feeding. One-third of the mixed urine was neutralized, evaporated, and extracted in the usual way. From this portion, 0.5 gm. of *p*-nitrobenzoic acid together with some unchanged *p*-nitrobenzyl cyanide was isolated.

After feeding 0.7 gm. of the substance to a rabbit on each of 4 successive days, it was found that the substance had little or no effect on metabolism.

*p*-Nitrobenzyl cyanide is almost non-toxic, due largely no doubt to its comparative insolubility.

#### *o*-Nitrobenzyl Cyanide.

This substance was prepared from *o*-nitrophenylacetic acid; its effect on metabolism was studied by keeping a rabbit on a carrot diet for a week to get the normal figures for the various nitrogen and sulfur compounds, and then injecting at intervals of 2 days 0.125, 0.4, 0.4, and 0.4 gm. of the substance. That it was rapidly absorbed and excreted was shown by the rise in the total nitrogen immediately following an injection. Little was converted into thiocyanates. There was a fairly marked rise in the amount of ethereal sulfates, and a positive Millon's reaction was obtained after the large injections. These two facts indicate an oxidation in the ring, as is to be expected of any aromatic compound not protected in the para position.

The *o*-nitrobenzyl cyanide was dissolved in olive oil and injected subcutaneously into a dog in 1 gm. doses. The urine was treated in the usual way, but the ethyl acetate extraction yielded no product. The color test with sodium hydroxide, however, always showed the presence of traces of the unchanged substance in the urine. After a subsequent experiment in which a total of 10 gm. were injected, the urine was acidified without evaporation and extracted in a separatory funnel with large quantities of ether; this was then allowed to evaporate and the residue was recrystallized from hot water. 0.5 gm. of *o*-nitrobenzoic acid was isolated.

The *o*-nitrobenzyl cyanide is one of the least poisonous of these substances, and in this respect it bears considerable resemblance to *o*-nitrophenylacetic acid, the least toxic of the substituted phenylacetic acids.

*Benzyl Cyanide.*

Benzyl cyanide was fed to a rabbit which had been kept on a carrot diet for 8 days while all the various forms of sulfur and nitrogen were determined. Then every 2nd day the substance was injected subcutaneously in doses of 0.1, 0.2, and 0.5 gm.; the animal died about an hour after the last dose. There was a slight increase in the thiocyanate excretion in the urine; there was also a positive Millon's reaction, and a doubling of the amount of the ethereal sulfate excreted, but the amount of the cyanide excreted as thiocyanate and ethereal sulfate would not account for more than 5 per cent of the amount fed.

A dog of 31 kilos was allowed an ordinary diet for 4 days, while nitrogen and sulfur partitions were run. He then received every 2nd day an injection of benzyl cyanide until four doses had been administered of 1.25, 2, 2, and 3 gm. respectively. The dog showed no marked signs of intoxication; as the experiment progressed he lost appetite but drank large quantities of water. Even after the 3 gm. dose, this compound had no decided influence on the endogenous catabolism, as was shown by an ever decreasing output of total nitrogen. Less than 3 per cent can be accounted for by thiocyanate excretion. Millon's reaction was consistently negative, but there was a small increase in the output of ethereal sulfates. Only about 5 per cent of the material can be accounted for by conversion into hydroxy cyanide or hydroxy acid. If such a conversion took place it would be represented by nuclear oxidation or hydrolysis or perhaps both.

A sample of each day's urine was made alkaline with sodium carbonate, evaporated to a thick syrup, and acidified to Congo red with sulfuric acid. The mixture was then extracted in a rotary extractor with ethyl acetate in the usual way. After decolorizing the water solution with charcoal, the liquid was gently evaporated on a water bath until crystals appeared. They were identified as benzoic acid. Small amounts were obtained after each feeding; the total was about 0.8 gm. No hippuric acid was ever found.

*p-Chlorophenyl Cyanide.*

After a preliminary observation period of 8 days, a rabbit was injected with the following doses of *p*-chlorophenyl cyanide dis-

solved in olive oil: 0.125, 0.15, 0.25, 0.33, and 0.75 gm. on 5 successive days. The substance caused no marked rise in total nitrogen or total sulfur excretion; there was no noticeable alteration in any of the nitrogen or sulfur constituents of the urine. The toxicity of the substance was not sufficient to cause any marked symptoms in the rabbit.

A large dog was given a total of 5.5 gm. of the cyanide in four doses on 4 successive days. The urine was treated in the usual manner. In a second experiment the same animal received 7 gm. in four doses. The urine was mixed, measured, and divided into three nearly equal parts. The first part was evaporated on the water bath and extracted with ether; it was then acidified and extracted with ether again. The second part was extracted with ether without evaporation; while the third part was treated with a 10 per cent solution of sodium nitrite and boiled under a reflux for an hour. By this treatment it was hoped that any *p*-chlorophenyl cyanide or *p*-chlorobenzamide present might be hydrolyzed to *p*-chlorobenzoic acid. In a third experiment the dog was again injected with two 1 gm. doses on 2 successive days. The urine was evaporated *in vacuo*, acidified, and extracted in the rotary extractor, first with ether, and then with ethyl acetate. In no one of all these experiments were more than traces of the *p*-chlorophenyl cyanide or the *p*-chlorobenzoic acid ever found. The substance was excreted partly in the feces and perspiration, and the odor of the breath indicated that a good deal was also eliminated through the lungs.

#### *Phenyl Cyanide.*

Two experiments on rabbits were made with phenyl cyanide. In the first the feedings were by subcutaneous injection and the animal died after the third dose; the amounts were 0.1, 0.2, and 0.25. In the second experiment, the phenyl cyanide, an oily liquid, was fed by means of a stomach tube in 0.1, 0.2, and 0.25 gm. doses as an emulsion in 50 to 100 cc. of water. An average 2 kilos rabbit was able to withstand five doses of the substance. There was a decrease in the urine volume, which generally follows poisoning by an aromatic cyanide, but which was perhaps the most marked in this case. No signs of intoxication were observed until about 4 hours after the last dose. After  $\frac{3}{4}$  of an hour con-

vulsions set in, and in an hour's time the rabbit appeared practically dead. Recovery was almost as striking as the illness. 12 hours after the first signs of discomfort the animal seemed perfectly normal. Millon's reaction was positive throughout the feeding, whereas in the first experiment it was positive only after the final dose of 0.25 gm.

A medium sized dog was injected with 13.2 gm. of phenyl cyanide. Some nitrogen and sulfur determinations were made on the urine before the feeding began. The urine, acid at first, became alkaline after the fourth injection. No positive Millon's reaction could be determined, nor did the urine ever give a test for thiocyanates with ferric chloride. Hippuric acid determinations were made, but when nothing was found after the first feeding, routine work was given up and all efforts were directed toward the isolation of any product that could be found in the urine. No benzoic acid, hydroxybenzoic acid, or hippuric acid was detected, and only small amounts of phenyl cyanide could be recognized in the residue.

#### *p*-Nitrophenyl Cyanide.

This compound was prepared by a Sandmeyer reaction from *p*-nitraniline; no suitable solvent for its injection could be found. A dog, fasted for 4 days, was induced on the 5th day to swallow a chunk of meat in which about a gm. of *p*-nitrophenyl cyanide, contained in a gelatin capsule, had been enclosed. The dog, a powerful animal of 40 kilos, died in convulsions within an hour. A sample of urine was found which gave negative indications with Millon's reagent and with ferric chloride; there was nothing abnormal about it except a faintly positive reaction for albumin. No attempt was made to feed the substance to rabbits.

#### *2,4*-Dichlorophenyl Cyanide.

This substance was prepared by a Sandmeyer reaction, following the specific directions of Gomberg. The oily substance obtained by steam distillation boiled at 215°. Two doses of 2 gm. each were injected into a dog subcutaneously. The urine was extracted with ether in a separatory funnel before and after acidification. No compound of a cyanide nature was found. With the excep-

tion of a trace of albumin, the findings were quite normal. The dog suffered little inconvenience.

#### SUMMARY.

The following substances have been fed to dogs and rabbits: phenyl cyanide, *p*-nitrophenyl cyanide, 2,4-dichlorophenyl cyanide, *p*-chlorophenyl cyanide; benzyl cyanide, *p*-nitrobenzyl cyanide, *o*-nitrobenzyl cyanide, and *p*-chlorobenzyl cyanide.

Of these the *p*-nitrophenyl cyanide is by far the most toxic, and *p*-nitrobenzyl cyanide, on account of its extreme insolubility, is the least toxic.

In general only small amounts of end-products were detected after feeding these substances. The detoxication mechanisms were quite diversified; slight conversion to the acid occurred in some cases, and in others an increase in ethereal sulfate or thiocyanate accounted for a small per cent of the material fed. When *o*-chlorobenzoic acid is fed, it is excreted as *p*-chlorohippuric acid, but the *p*-chlorobenzoic acid produced by the detoxication of *p*-chlorobenzyl cyanide is excreted unchanged.

The general processes of detoxication of benzyl and phenyl cyanides and their derivatives thus far studied do not occur by hydrolysis of the CN group either to the amide or the acid. Their toxicity is not proportional to the rate at which they split off HCN, but seems to be a highly specific property. In the case of the benzyl cyanides, the CN group is split off and the alpha carbon atom is oxidized to the acid.

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## THE RELATION OF IRON FROM VARIOUS SOURCES TO NUTRITIONAL ANEMIA.

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The time-worn question as to what form of iron can best be utilized for hemoglobin synthesis in the animal body is still unanswered despite the many contributions from various workers since the discussion was opened by Von Bunge and Abderhalden before the beginning of the present century.

Inorganic and medicinal iron remedies have been variously designated as absolutely ineffective, as merely stimulating, or as actually participating in hemoglobin building. Priority has also been claimed by some for certain types of food iron as especially efficient blood builders. Proof of these hypotheses has been vigorously sought and attacked from various angles. Although each contribution has added valuable knowledge, the problem as a whole is not yet solved.

Clinical observations on the use of iron in the treatment of anemia are too extensive to review in detail and moreover are often misleading and confusing because of extraneous influences which the writer has failed to take into consideration. Barkan (1), Baumgarten (2), and Seyderhelm (3) all report beneficial results from the use of large quantities of inorganic iron, but disagree as to its exact function.

Experimental work with animals concerning anemia has been mainly along two lines. McGowan and Crichton (4) and Scott (5, 6) record successful treatment of anemia in swine and rats by use of inorganic iron salts, but the blood picture has not been carefully followed, nor are the contrasts striking enough to be convincing.

Along another line of attack, Whipple, Robscheit-Robbins,



and Hooper (7) report an extensive piece of work showing the influence of various foods on regeneration of blood in severe anemia produced by hemorrhage.

In reviewing the various reports of work done it would seem that the regeneration after hemorrhage method affords opportunity for accurate comparative results, but falls short of solving the problem of, or simulating the naturally occurring anemias in, the human organism. Chronic anemia is not produced by a sudden loss of blood but by some deficiency in the nutrition or functioning power of the individual. It is therefore possible that factors which may aid in red cell formation under the former emergency conditions, where an extreme stimulus is present, may be somewhat different from those utilized in more chronic conditions. Moreover, much of the work reported has been limited in the scope of the iron compounds investigated. The results of different investigators, a few of which I have cited, carried on with inevitable differences in method and technique, are not satisfactory for comparative purposes.

In view of the previous discussion and with our own plan of work outlined and under way, we were much interested to read the results obtained by Hart, Steenbock, Elvehjem, and Waddell (8) in their work on nutritional anemia in rabbits. Their findings that ferric oxide alone cannot correct or prevent anemia, but that it is effective when accompanied by iron-free organic compounds such as chlorophyll, are in agreement with some previous hypotheses. Their conclusion, however, that "Inorganic iron ( $\text{Fe}_2\text{O}_3$ ) added to the basal ration will not *per se* correct this anemia," is too broadly interpreted, in view of our present observations. It does not seem that a single compound such as ferric oxide should be taken as representative of all inorganic iron compounds.

The observations presented in this paper were made on extremely anemic rats, before and after the addition of equal quantities of iron from widely varying sources, with the purpose of comparing availability.

Emphasis is placed on the fact that the anemia was not produced by hemorrhage but was truly of a nutritional origin and yielded to the alimentary administration of available iron from various sources, but persisted in the presence of less available forms.

## EXPERIMENTAL.

*Reproduction and Raising of Young Anemic Rats.*

In our experience we had found, as did Scott (6), that second generation rats on low iron diets such as milk or white bread and milk were extremely anemic and might afford material for interesting observation. We did not find, however, that the young recovered spontaneously on such diets within a period of 6 months. They frequently failed to live even that long unless some change was made in the diet, and were extremely anemic if they did survive. Our difficulty had always been that reproduction on milk diets was poor and mortality of the young high. This poor reproduction in anemic rats was to be expected and had probably been a contributing factor in the sterility noted by Mattill and Stone (9) and Anderegg and Nelson (10) in their reproduction experiments. Some improvement in fertility and lactation was noted when fresh celery tops were fed, but the hemoglobin remained low and the mortality of the young was still higher than that of our stock colony. The celery probably supplied additional vitamin E, but because of the low iron content the anemic condition persisted.

A suggested solution to our problem of reproduction on milk diets came in the report of Daniels and Hutton (11) showing the beneficial results obtained by adding traces of certain minerals to the milk. This effect was most evident in promoting better reproduction and survival of the young. The best results were obtained when all six minerals mentioned were added, but their findings indicated definite beneficial effects when only four or five of the salts were added. Consequently, since the production of anemic rats was our object, the iron was withheld but the five other salts of manganese, fluorine, silicon, aluminum, and iodine were added to the milk in the quantities as indicated by Daniels and Hutton (11).

Our records indicated a decided improvement in reproduction directly following the addition, but better weather conditions at that same period may have been a contributing factor. The fact remains, however, that sufficient young survived to permit starting an extensive series of experiments. The addition to the milk of the mineral solution as modified for our purpose became

a routine procedure for all of the milk-fed breeding stock, and later for all of the rats on experimental diets. About 120 rats have been raised in this way and used for the present series of experiments, including some utilized for preliminary tests not reported here.

#### *Care and Feeding of Experimental Rats.*

Young rats were put on experiment soon after weaning or as soon as blood for hemoglobin determinations could be drawn without injury to the animal. In the more stunted ones this was at the age of 6 weeks. All experimental animals were caged singly or in pairs in small galvanized wire cages with raised wire bottoms. Pans and bottoms were changed daily for sanitary reasons and to avoid consumption of excreta. Further precautions with respect to iron-free cages were found to be unnecessary since control rats on diets low in iron showed no tendency to spontaneous recovery. All rats were weighed twice a week and hemoglobin determinations made once a week.

The basal ration, as previously stated, was fresh whole certified milk (from cows that had no access to fresh pasture) plus the traces of minerals as suggested by Daniels and Hutton (11). All of the tests were based on curative experiments and not on preventive. The amounts of iron-containing supplements to be fed were calculated so that the daily allowance of iron would be 0.4 mg. This amount had been proven capable of effecting a rapid rise in hemoglobin if it was derived from an easily available source. Less than 0.4 mg. of iron was decidedly inadequate for stimulating recovery, as shown by several preliminary trials with different foods. A greater quantity, which might effect a quicker response, would nevertheless blot out the finer distinctions between different sources of iron, and in some cases was excreted unutilized as indicated by the color of the feces. By this procedure of keeping the quantity of iron minimal and constant it was our purpose to demonstrate the relative availability of iron from various sources.

The milk was always fed *ad libitum* and the weighed amount of iron-containing supplement added with the first feeding in the morning. More milk was given later in the day if all other food had been consumed. Account was kept where the rats

failed to eat their allotted iron ration. This difficulty was experienced where the food was a concentrated sweet or where a large quantity was necessary to supply the requisite 0.4 mg. of iron daily. Amounts of supplementary foods to be used were based upon the iron analyses recorded by Sherman (12) or upon special determinations made in our chemistry laboratory. Amounts of each food or salt used are recorded in Tables I, II, and III.

The sources of iron thus far investigated are as follows:

*Foods*.—Celery, egg yolk, meat, raisins, dates, and spinach.

*Medicinal*.—Ovoferrin (13).

*Inorganic*.—Ferric chloride, ferric ammonium citrate, ferric oxide, and ferrous carbonate.

#### *Hemoglobin and Blood Counts.*

Hemoglobin determinations were at first made by use of the Dare and Tallqvist scales, but later these were discarded for a more accurate method. All of the records reported in this paper are based on the acid hematin method of Cohen and Smith (14) with the standard made to correspond to that of Dare; *i.e.*, 13.77 gm. of hemoglobin to 100 cc. of blood. Dare and acid hematin determinations were carried parallel for a few weeks and found to check fairly well except in the very low percentages. That hemoglobin percentage determination is a true criterion for total hemoglobin in anemic rats is confirmed by Scott and Barcroft (15) who showed that blood volume remains normal even in very anemic rats.

Erythrocyte counts of anemic rats ranged from 1,330,000 to 4,900,000. Two rats 90 days of age showed counts of 1,330,000 and 2,200,000 in marked contrast to the count of 8,500,000 given by Donaldson (16) for normal rats of that age. The blood picture of some of the young rats, having a slightly higher erythrocyte count, was however poor compared with that for corresponding normal animals. The lower counts in the older rats would tend to refute the argument that there may be spontaneous recovery. 5,000,000 to 6,000,000 erythrocytes have been the maximum reached in most of our rats after the period on iron supplement. This is still below Donaldson's figures for normal rats but nevertheless our animals seemed to be in good healthy

condition and always much improved compared with the preceding period in which no iron supplement was furnished. Microscopic examination of the stained cells showed them to be mature and well formed.

*Method of Bleeding.*

A freely flowing drop of blood was obtained from the tip of the rat's tail by gently stroking to increase the circulation. In young rats or where blood came with difficulty, the device of holding the tail in warm water for a moment and then drying with a towel before clipping the end was found most effective for increasing the circulation temporarily and gave no evidence of permanent hyperemia in the region. Since our experimental anemia was not produced by hemorrhage the blood volume was not reduced and therefore the amount of circulating blood in the tail would seem to warrant this method of bleeding.

Criticism of the procedure as offered by Robscheit-Robbins and Whipple (7) would be in order if the differences noted were small, for there are undoubtedly some difficulties in the method. The careful technique developed and the fairly constant and consistent results observed, however, make us feel confident that the blood obtained was a fair sample of the circulating fluid. Questionable results due to recognized difficulty in obtaining a fair sample of blood or other technical errors were carefully recorded and accordingly discounted in drawing conclusions.

Throughout the experiment an attempt has been made to keep the rats in as normal a condition as possible and unfrightened. To accomplish this, all mechanical devices for holding the rats during bleedings were avoided. During the procedure the rat was held by one person, the tail manipulated by a second, while a third drew the blood for the test. The rats became so accustomed to the performance that they seldom resisted or attempted to bite the helpers. One or two obstreperous ones were discarded for this reason.

DISCUSSION.

Out of every litter of anemic rats started on experiments, one or two have remained on milk alone without iron supplement for

control purposes, but we can show comparatively few hemoglobin determinations on such rats, for the mortality was extremely high. Hemoglobin figures on 85 such rats ranged from 20 to 50, averaging 30.5 per cent. The pale color of the eyes and ears was striking to even the untrained observer. In this condition the rats

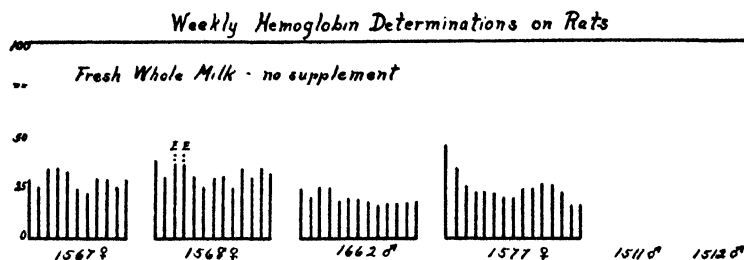


CHART I.

TABLE I.  
*Response of Rats on Fresh Whole Milk without Iron Supplement.*

Supplement furnish- ing iron.	Rat No.	Age at start.	Weight at start.	Weight after 90 days.	Hemoglobin.				Erythro- cyte count after period on milk.
					At start.	After 30 days.	Maximum in:		
							60 days.	90 days.	
None.		days	gm.	gm.	per cent	per cent	per cent	per cent	
	1567 ♀	38	50	150 (85)*	28	30	32		
	1568 ♀	38	48	142 (85)*	42	32	36		
	1662 ♂	47	62	132	24	20	20	20	1,330,000
	1577 ♀	36	54	149	49	22	24	24	2,200,000
	1511 ♂	37	32	69 (37)*	35	25			
	1512 ♂	37	34	80 (37)*	35	30			
	1799 ♀	37	34	48 (21)*	22	22			2,020,000
	1806 ♀	45	71	135 (42)*	32	36			4,210,000
	1819 ♀	30	61	140 (42)*	53	40			4,890,000

\* Figure in parenthesis indicates number of days if less than 90.

had very little resistance and showed extreme susceptibility to changes in temperature. On a few occasions when the thermostatic device in the laboratory failed to function there was a drop of from 10-15° in the temperature below the usual 72°F. None of the 300 rats in the room showed ill effects from this drop except the anemic ones, of which five at one time and three at another were

found dead in the morning. In most of the cases we tried to watch the rats and change the diet before they were too weak to recover. In a few cases only have we been able to keep anemic rats on milk alone for as long as 12 to 15 weeks. The hemoglobin records of six control rats given in Chart I are representative of the whole group and were chosen because they were the ones which persisted for the longest periods on milk alone. These same rats were used for other tests following the period on milk. In no case did we find a spontaneous recovery or increase in hemoglobin or erythrocytes while on milk diet.

Growth records of these anemic rats show no loss of weight but rather a slow steady gain much below the normal. There was no evidence of any vitamin deficiency in any of our experimental rats.

When an iron supplement was to be added to the diet of young rats, blood tests were always made the day preceding the change and at weekly intervals thereafter. The charts indicate the hemoglobin percentage weekly. Only those foods having rich iron content could be successfully tested, as has been previously stated. Such food as celery, for instance, containing 0.0005 per cent of iron would have to be eaten in 80 gm. quantities daily to supply the requisite 0.4 mg. of iron. In no case would the rats eat more than one-fourth of this amount and consequently showed very poor hemoglobin response, more from deficiency in quantity than quality, probably. Experiments on celery are, therefore, not reported in detail in this paper.

Somewhat the same difficulty was experienced with fresh raw spinach of which 11 gm. daily were required. The difficulty in this instance was overcome, however, by drying the spinach and mixing the dry powder with their daily milk ration. Previous workers (8) have noted that drying of cabbage leaves does not interfere with hemoglobin-building properties. Only 1.54 gm. of the dry New Zealand spinach were needed to supply the necessary iron. The use of the broad leaf variety was necessitated by change in season but record of this is made in every case. New iron determinations could not be made immediately, therefore we continued feeding the same quantity. The slight stimulus resulting from the change is probably due to the higher iron content of the broad leaf spinach. The slow response of Rats

*Weekly Hemoglobin Determinations on Rats*  
*Diet: Milk plus Various Iron-Containing Supplements*

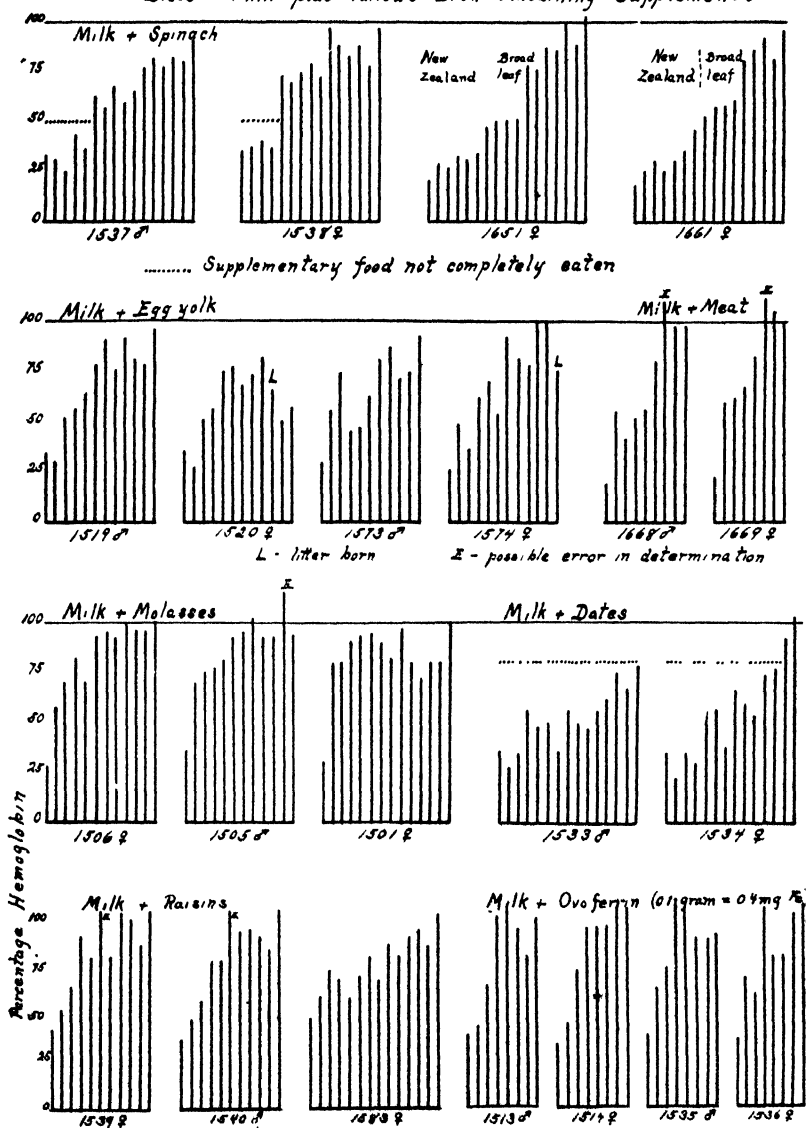


CHART II.



TABLE II.  
*Response of Rats to Iron Supplement.*

Supplement furnishing 0.4 mg. of Fe.	Rat No.	Age at start.	Weight at start.	Weight after 90 days.	Hemoglobin.				Erythro- cytes, maximum.
					At start.	After 30 days.	Maximum in:		
							60 days.	90 days.	
		days	gm.	gm.	per cent	per cent	per cent	per cent	
Spinach (dry), 0.026 per cent Fe; 1.54 gm. fed daily.	1537	26	37	215	38	43	66	80	5,800,000
	1538	26	40	157	39	50	75	100	5,320,000
	1651	47	70	195	23	32	50	82	5,560,000
	1657	53	46	248	18	47	97	112	5,960,000
	1661	33	53	220	22	30	58	83	5,760,000
	1672	49	61	183	27	77	77	97	5,570,000
Egg yolk, 0.0086 per cent Fe; 4.6 gm. fed daily.	1519	37	36	250	35	65	92	98	5,500,000
	1520	37	34	185	37	73	83	*	5,700,000
	1573	38	38	250	33	77	88	88	5,690,000
	1574	38	44	190	28	70	92	100	5,720,000
Molasses, 0.0073 per cent Fe; 5.5 gm. fed daily.	1501	46	42	152	35	92	98	100	5,600,000
	1505	25	27	207	35	84	103	117	6,000,000
	1506	25	30	156	27	84	100	109	5,520,000
Dates, 0.003 per cent Fe; 13.3 gm. fed daily.	1533	53	90		27	57	57	77	5,710,000
	1534	53	70	230	27	58	64	77	5,450,000
Raisins, 0.0021 per cent Fe; 19 gm. fed daily.	1539	26	40	180	35	87	100	102	5,600,000
	1540	26	37	180	39	78	102	102	5,550,000
	1582	36	53	250	43	57	57	70	5,940,000
	1583	36	53	157	43	72	77	90	5,930,000
Meat (dried), 0.013 per cent Fe; 3.1 gm. fed daily.	1668	47	46		20	55	112	115	6,300,000
	1669	47	47	155	23	67	115	115	5,750,000
Ovoferrin, 0.1 gm. fed daily = 0.4 mg. Fe.	1502	25	26	153	40	75	108	108	5,600,000
	1503	25	25	140	40	75	100	100	
	1513	37	35	168	32	98	98	98	5,650,000
	1514	37	30	137	32	93	100	105	5,680,000
	1535	26	45	207	37	112	112	112	5,760,000
	1536	26	34	155	40	100	103	103	5,380,000
	1569	38	32	114	32	70	82	97	
	1570	38	49	170	30	105	112	112	
	1578	36	53	225	50	89	108	108	5,600,000
	1579	36	47	150	50	88	125	125	6,100,000
	1665	54	57	190	45	97	103	112	5,650,000

\* Litter.

1537 and 1538 during the first 6 weeks of the experiment was due partly to difficulty in inducing them to eat the spinach but better response was noted after complete consumption was insured, as well as when a change to the other variety was made.

Another unforeseen difficulty was encountered in the feeding of dates and raisins of which 13.3 and 19 gm. respectively were necessary to provide the 0.4 mg. of iron. The concentrated sweet seemed to be objectionable and it was only after grinding and mixing with the milk that the rats could be induced to eat even the larger portion of their ration. We were never able to get them to eat the full amount of raisins or dates consistently every day, and the hemoglobin response was consequently delayed. There was nothing to indicate that the iron from these sources is not in available form since the blood responded as soon as more of the food was eaten. This observation confirms our previous contention that less than 0.4 mg. of iron daily was insufficient for rapid recovery. Growth on the raisin and date diets was practically normal (Chart II, Table II).

The excellent growth and hemoglobin response during the first weeks on the egg yolk experiment were remarkable but the slowing up in hemoglobin rise later suggested that the 0.4 mg. of iron was insufficient in quantity for the latter part of the rapid growing period. No change in amount of supplementary iron, however, was made in this experiment but further work is planned to check this quantity relationship by additional increments of iron to be made at certain intervals (Chart II, Table II).

When high grade New Orleans molasses furnished the supplementary iron the growth was nearly normal in all cases and the rise in the amount of hemoglobin was sharp. The slight fall in the quantity of hemoglobin a little later may be suggestive of the same situation as mentioned in the case of egg yolk; namely, that the quantity of iron supplied was insufficient for the larger rats. The 5.5 gm. fed daily were based on the iron content of molasses as given by Sherman. Since molasses varies greatly in its mineral content the exact amount of iron obtained by the rats was uncertain (Chart II, Table II.)

Less extensive observations have been made on meat as a source of iron but the excellent growth and blood picture would indicate that the iron from this source must be easily available.

Ovoferrin is an organic preparation popularly used in the clinical field and was investigated along with the food iron and iron salts for comparative purposes. According to the label on the bottle 0.1 gm. would contain 0.4 mg. of iron, and it was used in that quantity. The rapid hemoglobin response always observed on the product would indicate that the iron was in an easily available form. A slight irregularity later might be explained as due to deficient quantity as previously mentioned.

The inorganic iron salts chosen were either representative of those used in various tonics or showed extreme limits of solubility. Ferric chloride and ferric ammonium citrate are readily soluble in cold water, while ferric oxide and ferrous carbonate are insoluble. These salts were each fed in quantities which would furnish our established standard of 0.4 mg. of iron daily. Any difficulty in insuring the complete consumption of the less soluble compounds was overcome by feeding them with a very small quantity of milk first and allowing the rats to lick the dish clean before other milk was given. In the case of the ferric oxide, which would not even stay in water suspension long enough to be accurately measured, we found that if made up in a medium thick starch paste an even distribution of material was obtained and this mixture could be kept for several days in an ice chest. The response on both the oxide and carbonate was decidedly slower, more irregular, and in some cases almost nil compared with the rapid recovery in the blood picture of the rats given the chloride and citrate. A temporary rise in quantity of hemoglobin noted in a few of the rats on the oxide and carbonate test was attributed to possible error in technique or feeding, or perhaps to slight utilization (Chart III, Table III).

The question as to whether ferric oxide and ferrous carbonate could have been better utilized if accompanied by chlorophyll as suggested by Hart and Steenbock (8) has also been investigated in this series of experiments. In no case did the presence of the chlorophyll affect in any apparent way the availability of iron from the oxide or the carbonate.

According to our observations, some soluble types of inorganic iron as illustrated by the chloride and the citrate can be utilized for the building of hemoglobin without the presence of any chlorophyll-like compound other than that which could possibly be

## Weekly Hemoglobin Determinations on Rats

Diet: Milk plus Inorganic Iron (0.4 mg. Fe daily)

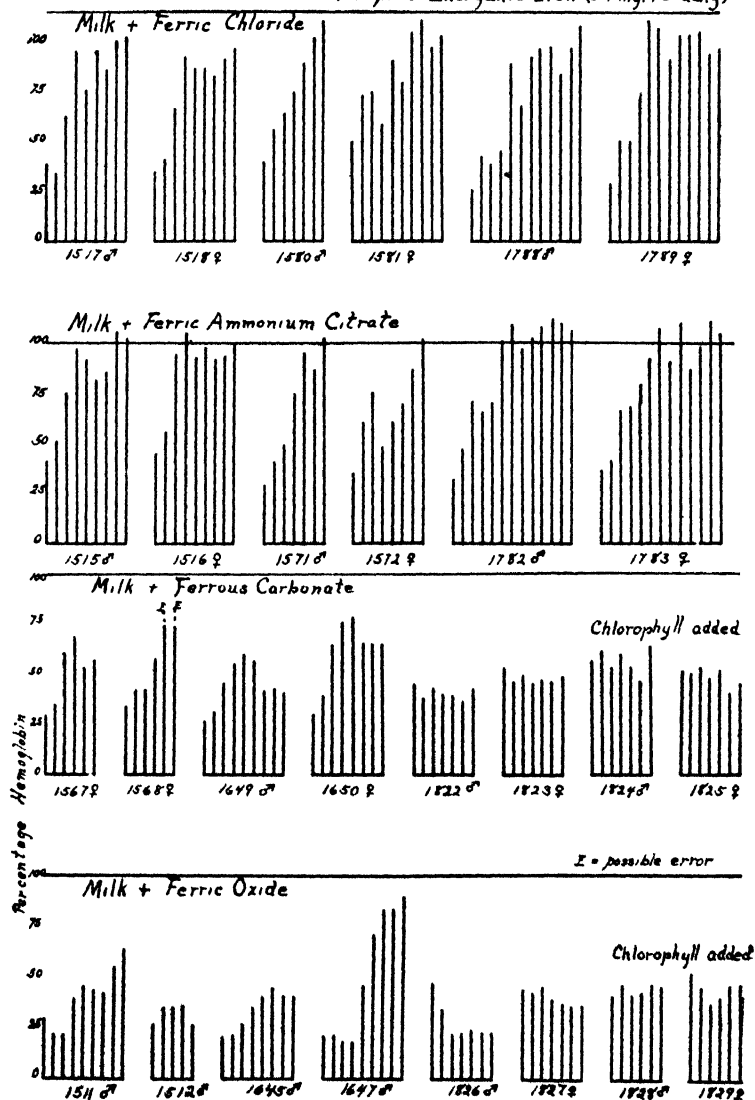


CHART III.

TABLE III.  
*Response of Rats to Inorganic Iron Supplements.*

Supplement furnishing 0.4 mg. of Fe.	Rat No.	Age at start.	Weight at start.	Weight after 90 days.	Hemoglobin.				Erythro- cyte count after period on special diets.
					At start.	After 30 days.	Maximum in:		
							60 days.	90 days.	
		days	gm.	gm.	per cent	per cent	per cent	per cent	
Ferric chloride, mol. wt. 270; 1.9 mg. fed daily.	1517 ♂	37	35	140	35	93	105	105	6,000,000
	1518 ♀	37	28	117	33	93	95	97	5,760,000
	1580 ♂	36	49	182	38	90	107	107	
	1581 ♀	36	48	160	50	92	112	112	5,600,000
	1788 ♂	49	47	190 (77)*	26	112	112		5,720,000
	1789 ♀	49	75	160 (77)*	26	89	98		5,470,000
Ferric ammonium citrate, mol. wt. 579.7; 2.1 mg. fed daily.	1515 ♂	37	35	215	37	90	107	107	6,130,000
	1516 ♀	37	38	168	45	102	102	102	5,740,000
	1571 ♀	38	48	177	30	97	112	112	5,790,000
	1572 ♀	38	42	155	33	78	117	117	5,610,000
	1782 ♂	32	47	260	33	71	101	101	5,480,000
	1783 ♂	32	37	200	37	81	93	111	4,960,000
Ferrous carbonate, mol. wt. 139.8; 0.83 mg. fed daily.	1567 ♀	123	150	185	28	72	89	102	5,830,000
	1568 ♀	123	145	175	35	92	98	98	5,710,000
	1649 ♂	69	115	210	23	58	60	60	5,200,000
	1650 ♀	69	113	160	30	82	88	90	6,300,000
	1784 ♂	32	41	168	35	33	33		2,280,000
	1785 ♂	32	38	130	37	37	52		3,170,000
	1822 ♂	30	59	150 (42)*	48	40			4,600,000
	1823 ♀	30	56	126 (42)*	55	47			5,060,000
	1824 ♂	30	63	150 (42)*	57	55			5,070,000
Same amount plus chlorophyll.	1825 ♀	30	54	125 (42)*	51	52			5,920,000
Ferric oxide, mol. wt. 162; 0.57 mg. fed daily.	1511 ♂	74	68	150 (60)*	25	50	65		
	1512 ♂	74	80	190 (60)*	30	35	35		
	1645 ♂	56	85	250	22	37	48	68	6,050,000
	1647 ♂	53	86	200	21	48	93	105	5,870,000
	1790 ♀	49	57	125 (49)*	22	68			5,400,000
	1791 ♀	49	57	130 (49)*	24	56			5,600,000
	1826 ♂	30	64	90 (35)*	50	22			4,750,000
	1827 ♀	30	51	106 (35)*	45	37			5,380,000
	1828 ♂	30	62	135 (35)*	43	45			5,100,000
Same amount plus chlorophyll.	1829 ♀	30	69	132 (35)*	53	43			4,930,000

\* Figure in parenthesis indicates number of days if less than 90.

present in the milk. The same milk was used, however, with the insoluble compounds where poor utilization was observed. We therefore conclude that there is no particular significance in the presence of chlorophyll in influencing the availability of iron.

The foregoing results seem to justify the conclusion that at least some forms of inorganic iron are utilized by the animal organism to build hemoglobin and not merely absorbed and stored as suggested by Williamson and Ets (17). Our observations are suggestive of a new line of differentiation between available and unavailable sources of iron; namely, *soluble versus insoluble*, rather than *organic versus inorganic*.

#### SUMMARY.

A true nutritional anemia can be produced in rats raised from parents that have been fed on diets low in iron.

When milk is used as the basic diet, the addition of traces of manganese, fluorine, silicon, aluminum, and iodine, as suggested by Daniels and Hutton, has seemed to promote better reproduction without affecting the anemia in the young rats.

In order to make a comparison of the availability rather than quantity of iron in each source, the amounts of each supplement fed have been so calculated as to furnish as nearly as possible 0.4 mg. of iron daily.

This quantity of 0.4 mg. of iron daily was chosen as enough to promote a rapid rise in hemoglobin when well utilized and was not enough to blot out finer distinctions in availability.

Erythrocyte counts when made corroborated the hemoglobin findings but in general showed a somewhat slower response. Hemoglobin determinations were used as the chief criteria in this series of experiments.

Iron from food and other organic sources showed availability as follows:

*Very good.*—Molasses, meat, and ovoferrin.

*Good.*—Egg yolk and spinach.

*Uncertain because of incomplete consumption.*—Raisins and dates.

Of the inorganic sources tested a definite line of differentiation occurred between the soluble and insoluble salts as regards their availability in the animal organism, as follows:

*Very good.*—Ferric chloride and ferric ammonium citrate.

*Poor.*—Ferric oxide and ferrous carbonate.

In view of these observations it is suggested that a new line of differentiation be drawn as regards availability of iron in the animal organism; namely, *soluble versus insoluble* rather than merely *organic versus inorganic*.

We wish to express our appreciation to Dr. W. B. Lewis and Mr. R. P. Bond of the Chemistry Department of the Battle Creek Sanitarium for preparing hemoglobin standards and making iron analyses on foods, to the Pathology Department for making erythrocyte counts, and to several of the students of Battle Creek College for assistance in the care and bleeding of the animals.

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## A COLORIMETRIC METHOD FOR THE DETERMINATION OF ACETONE BODIES IN BLOOD AND URINE.

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(Received for publication, August 17, 1926.)

The following paper describes a colorimetric method for the determination of acetone based on the reaction between acetone and salicylic aldehyde in alkaline solution with the formation of the colored product, dihydroxydibenzene acetone. The qualitative test for acetone which was introduced by Frommer in 1905 (1), based on this reaction, was modified by Engfeldt (2), who suggested a roughly quantitative method for clinical use, in which the urine is distilled and the distillate is diluted until its color with the aldehyde matches the color of one of a graded series of pure acetone solutions. In 1916 a somewhat similar method for the determination of acetone in urine was suggested by Csonka (3). The method is not adapted to the determination of small amounts of acetone. Large volumes of urine are required and the final determination is made in a colorimeter against a single standard containing forty times the minimum amount of acetone which gives a positive reaction according to Engfeldt. Like the earlier investigators Csonka used an alcoholic solution of salicylic aldehyde. His paper includes a suggestion for the determination of  $\beta$ -hydroxybutyric acid with the same reagents, after its oxidation to acetone by the Shaffer method. The paper does not include figures for any of the results obtained.

The present method attempts to adapt the salicylic aldehyde reaction to the determination of such small amounts of acetone as are found in normal urine and blood. It provides for the determination of 0.005 mg. of acetone in 5 cc. of distillate, so that with the dilution necessary for complete distillation a determination can be made using 2 cc. of blood, if the blood contains not less than 0.001 per cent of acetone. Greater accuracy is attained, however, if larger amounts are used.



$\beta$ -Hydroxybutyric acid is oxidized during a second distillation with potassium bichromate and sulfuric acid, as in the method first proposed by Shaffer (4) and modified by Hubbard (5). There are substances in urine which increase the color given by this second distillate in the final determination, and these are removed with copper sulfate and calcium hydroxide previous to the distillation, as suggested by Van Slyke (6). This treatment is not necessary in blood filtrates. In the procedure suggested by Csonka (3) the salicylaldehyde is added in alcoholic solution in the presence of sodium hydroxide, and the tubes are heated for 20 minutes in water kept at 45–50°C. In the present method the salicylaldehyde is added undissolved, and the tubes are heated in boiling water for from 3 to 5 minutes.

Acetone cannot be determined directly in urine or in blood filtrates with accuracy by this method.  $\beta$ -hydroxybutyric acid and acetoacetic acid both react to some extent under these conditions, glucose gives a distinct increase in the final color, and some urines and blood filtrates contain substances which apparently interfere with the formation of color.

#### *The Method.*

##### *Reagents.*

*For the Removal of Sugar and Other Interfering Substances by the Van Slyke Method (with Modified Concentrations).—*Copper sulfate, 40 per cent solution, or powdered in a mortar. Calcium hydroxide, 20 per cent suspension, or dry.

*For the Oxidation of  $\beta$ -Hydroxybutyric Acid by the Shaffer-Hubbard Method.—*50 per cent concentrated sulfuric acid. 0.2 per cent potassium bichromate solution.

*For the Removal of Blood Proteins by the Folin-Wu Method.—*10 per cent sodium tungstate solution.  $\frac{2}{3}$  normal sulfuric acid.

*For the Colorimetric Determination of Acetone.—*Sodium hydroxide, 32 per cent solution. Salicylic aldehyde.

We have found great differences in the delicacy of the color reaction given by different samples of salicylaldehyde. A sample of Kahlbaum's technical salicylaldehyde proved very satisfactory, as did also Eimer and Amend's high grade product labelled "Acid salicylous, synthetic, (salicylic aldehyde)." Samples from the Eastman laboratory, though less deeply colored than the

Eimer and Amend product, did not yield nearly so deep a color with a given amount of acetone as did the other samples used.

*Standard Acetone Solutions.*

*Stock Solution.*—This contains 0.1 mg. of acetone per cc. It is most easily prepared from a solution containing 1 cc. of acetone in 1 liter of water, whose actual acetone content by weight has been determined by an iodine titration.<sup>1</sup> The stock solution should be prepared from this to contain 0.1 mg. of acetone per cc. This solution can be kept for about a month without deterioration.

*Standard Solution.*—By 1:10 dilution of the stock solution a standard solution, containing 0.01 mg. per cc., is prepared for use in the actual determination. It is best to make up this dilute solution every 2nd day, and to keep it well corked when not in use.

*Determination of Acetone Bodies in Urine.*

*Preformed Acetone and Acetone from Diacetic Acid.*

(A) If  $\beta$ -hydroxybutyric acid is not to be determined:

Such a volume of urine as will contain about 0.1 mg. of acetone (usually from 2 to 50 cc. as required), is transferred to a 100 or 150 cc. distilling flask, the volume made up to about 75 cc. with distilled water, and 3 or 4 drops of sulfuric acid, diluted 1:1, are added. The flask is tightly fitted with a cork stopper and connected with a water-cooled condenser. The condenser is provided with a bent glass tube which has been drawn out long enough and to a sufficiently small diameter to reach to the bottom of a 25 or 50 cc. volumetric flask, and which dips below the surface of a minimum amount of water in the flask. None of the connections should be of rubber. Rubber stoppers covered with tin-foil, or cork stoppers, often renewed, can be used. The preformed acetone and acetone from diacetic acid are then distilled into the 25 or 50 cc. flask. Except when very large amounts of acetone are present a distillation to 25 cc. gives good results. When the distillate has almost reached the volume desired, the

<sup>1</sup> Sutton, F., Volumetric analysis, London and Philadelphia, 10th edition, 1911, 129, 384.

bent tube is disconnected and washed out with a few drops of water, and the distillate made up to volume and mixed. 5 cc. of the distillate are transferred to a test-tube and exactly 5 cc. of a 32 per cent solution of sodium hydroxide and 10 drops of salicylic aldehyde are added. Standards are prepared in test-tubes at the same time from the dilute (0.01 mg. per cc.) acetone solution. By using from 0.5 to 5 cc. of this solution a range of standards containing from 0.005 to 0.05 mg. can be made. Unless the approximate acetone content of the unknown solution is known, standards containing 0.005, 0.01, 0.02, 0.03, and 0.05 mg. had best be made. In each case the volume of the standard solution must be made to 5 cc. Exactly 5 cc. of 32 per cent sodium hydroxide and 10 drops of salicylic aldehyde are also added to each of the standard tubes. The contents of the tubes are mixed by side to side shaking and the tubes then immersed in a boiling water bath for from 3 to 5 minutes. If the salicylic aldehyde does not dissolve easily the tubes must be shaken until solution is effected. After the heating the tubes are removed and allowed to cool, the solutions filtered, and colorimetric comparison is made. The standard used should be of such a concentration that the unknown solution gives a reading between 11 and 19 mm. with the standard set at 15 mm.

In making the calculation the following formula may be used:

$$\frac{x}{y} \times \frac{p}{s} \times \frac{t}{5} \times 100 = \left\{ \begin{array}{l} \text{No. of gm. of acetone in 100 cc.} \\ \text{of blood or urine,} \end{array} \right.$$

$x$  = reading of standard.

$y$  = reading of unknown.

$p$  = gm. acetone in standard.

$s$  = cc. of blood or urine used.

$t$  = cc., volume of total distillate.

(B) If  $\beta$ -hydroxybutyric acid is also to be determined:

If  $\beta$ -hydroxybutyric acid is to be determined in addition to acetone, sugar and other interfering substances must be removed, even from normal urine, before any distillation is made. For this the urine is treated with copper sulfate and calcium hydroxide according to the Van Slyke procedure (6). In order to keep down the volume of solution to be distilled, however, the urine is diluted 1:5 instead of 1:10, using 1 volume of urine, 1 volume of 40 per cent copper sulfate solution, and enough of a 20 per

cent suspension of calcium hydroxide to make the reaction alkaline to litmus (probably 1 volume). The whole mixture is then made up to 5 volumes. If the urine is very low in acetone bodies the copper sulfate can be powdered in a mortar and both this and the calcium hydroxide added in powdered form. The copper sulfate should be dissolved before the calcium hydroxide is added. The mixture must be shaken very thoroughly and allowed to stand for  $\frac{1}{2}$  or  $\frac{3}{4}$  of an hour, with occasional shaking. It is then filtered and a volume of the filtrate equivalent to from 2 to 50 cc. of urine (depending on the acetone content) placed in a 300 cc. distilling flask and made acid with 3 or 4 drops of sulfuric acid (diluted 1:1). The volume is made up to about 75 cc., the flask fitted with a dropping funnel, and connected with a water-cooled condenser, and the distillation and determination of acetone and diacetic acid carried out as described under (A).

*Oxidation of  $\beta$ -Hydroxybutyric Acid by the Hubbard-Shaffer  
Method and Its Determination as Acetone.*

After distillation of the preformed acetone, a 100 cc. receiving flask is substituted for the 25 cc. flask, the residue in the distilling flask is brought to a boil, and 30 cc. of half concentrated sulfuric acid and 20 cc. of 0.2 per cent potassium bichromate are added gradually through the dropping funnel while a slow distillation goes on. 50 cc. more of the bichromate are added after 10 minutes and 50 cc. more after another interval of 10 minutes. The process differs from the Hubbard method only in that the distillation is made very slowly and the volume of distillate kept down to 100 cc. The distillation should occupy at least 30 minutes. When the distillation to 100 cc. is almost complete the receiving apparatus is again disconnected, the bent tube washed down with a little water, and the distillate made up to 100 cc. and mixed. Acetone is determined colorimetrically in 5 cc. of the distillate as described above under (A).

*Determination of Acetone Bodies in Blood.*

*Preformed Acetone and Acetone from Diacetic Acid.*

The blood proteins are precipitated by the regular Folin-Wu method (7), making a dilution of the blood of 1:10. From 10

to 100 cc. of the filtrate, depending on the acetone content, are transferred to a 300 cc. distilling flask, 3 or 4 drops of concentrated sulfuric acid, diluted 1:1, added, the volume made up to 50 to 75 cc., and distillation carried out as described for the determination in urine. The distillation is made into a 20 cc. receiving flask or graduated test-tube unless the acetone content of the amount of blood used is known to be high (above 0.05 mg.), in which case the distillation is made into a 25 cc. flask or graduated tube. If the amount of acetone in the filtrate used is known to be 0.1 mg., or more, the distillation may be made to 50 cc. In any case the distillation is stopped just before the desired volume has been reached, the distillate is made up to volume, and 5 cc. of the distillate are heated with alkali and salicylic aldehyde, cooled, and read in a colorimeter as described for the urinary determination.

*Determination of Acetone from  $\beta$ -Hydroxybutyric Acid in the Blood.*

1. If the actual volume of filtrate used is known to contain 0.1 mg. of  $\beta$ -hydroxybutyric acid as acetone, or more, the distillation is carried out as described for the determination in urine, the volume of the distillate being kept within 100 cc., and finally made up to 100 cc., and the colorimetric determination being made upon 5 cc. of the distillate, as described above under (A).

2. If the volume of filtrate used is expected to contain less than 0.1 mg., the distillation is carried out without regard to the volume of distillate collected, and after the 30 minute period, the distillate is redistilled into a 20, 25, or 30 cc. volumetric flask (or graduated test-tube), according to the amount of acetone expected. In any case the distillate is made up to volume and the acetone content of 5 cc. of the distillate determined as described above.

The formula given in the section on urinary determination under (A) may be used for all the calculations.

Tables I and II give figures for the recovery of acetone and  $\beta$ -hydroxybutyric acid from urine and blood by the new method and Table III gives figures for acetone by the new method as compared with figures given by the iodometric method.

TABLE I.

*Recovery, by the New Method, of Acetone and  $\beta$ -Hydroxybutyric Acid Added to Urine.*

All results are expressed as mg. of acetone per 100 cc. of urine.

Specimen.	Pre-formed acetone and diacetic acid from the urine.	Acetone added.	Added acetone recovered.	$\beta$ -hydroxybutyric acid from the urine.	$\beta$ -hydroxybutyric acid added.	Added $\beta$ -hydroxybutyric acid recovered.
Normal human.....	0.13	0.25	0.29			
" " .....	0.18	1.00	1.06			
" " .....	0.16	1.00	0.92			
" " .....	0.43	1.00	0.97			
" " .....	0.45	2.00	1.85	1.70	4.56	4.18
" " .....	0.38	3.77	3.48	1.97	9.12	8.92
" " .....	0.40	3.77	3.54	0.74	9.12	9.80
" " .....	1.50	3.77	3.84	3.20	8.00	7.90
Diabetic human*.....	3.12	2.00	1.82			
Dog (phlorhizinized)...	19.60	7.54	7.16			

\* We are indebted to Dr. Edward Tolstoi for supplying us with diabetic urines and bloods.

TABLE II.

*Recovery, by the New Method, of Acetone and  $\beta$ -Hydroxybutyric Acid Added to Blood.*

All results are expressed as mg. of acetone per 100 cc. of blood.

Sample.	Pre-formed acetone and diacetic acid from blood.	Acetone added.	Added acetone recovered.	$\beta$ -hydroxybutyric acid from the blood.	$\beta$ -hydroxybutyric acid added.	Added $\beta$ -hydroxybutyric acid recovered.
Beef.....	0.08	0.075	0.090	1.80	7.91	7.99
" .....	0.11	0.075	0.074			
" .....	0.21	0.754	0.910	1.86	0.47	0.50
" .....	0.32	0.188	0.170			
" .....	0.33	0.377	0.347			
Dog (phlorhizinized)...	3.24	1.88	1.66			
" " ...	6.15	0.75	1.01	6.86	4.00	4.10
" " ...	6.15	11.31	11.23	6.86	8.00	8.14

TABLE III.

*Comparison of Urinary Acetone in the Same Distillate by Iodine Titration and Salicylic Aldehyde Determination.*

Expressed as mg. of acetone per 100 cc. of urine.

Specimen.	By iodine titration.	By salicylic aldehyde reaction.
Dog (phlorhizinized).....	13.50	13.30
" " .....	19.52	19.07
" " .....	20.30	19.60
" " .....	20.40	19.45
" " .....	26.92	26.39
" " .....	30.45	30.90
Human diabetic.....	16.90	16.70
" " .....	18.80	17.56
" " .....	27.84	27.89

## SUMMARY.

A colorimetric method is described for the determination of the acetone bodies in normal or pathological urine or blood, based on the reaction of acetone with salicylic aldehyde in alkaline solution.

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## THE NUTRITIVE VALUE OF INORGANIC SUBSTANCES.

### I. A STUDY OF THE NORMAL ZINC METABOLISM WITH PARTICULAR REFERENCE TO THE CALCIUM METABOLISM.

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Until within comparatively recent times the occurrence of minute amounts of certain elements in plant or animal tissues has commonly been supposed to be the result of accidental contamination. As more and more light has been thrown on the subject, however, there has been a growing feeling that these elements may not be accidental in nature, but actually necessary for life. The relatively large amounts of zinc and its constant presence in all living tissue point to a necessary and definite utilization. The work of Bertrand (1), Javillier (2), and Delzenne (3) has done much to emphasize the importance of this metal in the functional activity of living cells.

Certain metals, although widely distributed, are seldom found in plant or animal tissues, while other metals are found in relative abundance compared with the amounts found in soil or water. An illustration of this selective action is the localization of iodine in certain types of seaweed. It is a curious fact that nature has roughly separated in this way toxic from non-toxic substances. Thus, in normal foodstuffs there are found iron, zinc, and manganese in about the same amount, whereas definitely toxic elements such as lead, arsenic, or mercury are either wholly absent or are present, as in the case of arsenic, in exceedingly minute amounts compared with these other non-toxic substances. The common occurrence of such metals as zinc and manganese led Javillier, Bertrand, and their associates to investigate life conditions in which these elements were lacking. Their respective investigations definitely established the necessity of manganese for the



growth of *Aspergillus niger* and zinc for the normal growth of white mice. So far, however, no study has been made of the conditions affecting the use of these metals in the body.

Inasmuch as it is very difficult to free foodstuffs from extremely small amounts of inorganic substances without extensively denaturing the food, the present problem was attacked in another manner. The association of calcium excretion with that of several other metals such as magnesium (4, 5), sodium and potassium (5), and lead (6) indicates the desirability of correlating the behavior of mineral substances in the body. The conditions were therefore determined under which zinc was retained or moved about in the organism and its relation in these respects to the calcium metabolism. An investigation of the amount of zinc present in the blood of various animals demonstrated a definite and somewhat constant quantity. This fact in itself points to a definite utilization in the body for some as yet undefined purpose. Otherwise, the very efficient threshold established by the organism towards toxic substances would function in preventing the entrance of zinc salts into the blood stream. The results of the present investigation indicate that zinc can be moved freely from the body, so that the normal tenacious retention in the blood can hardly be looked upon as accidental.

#### EXPERIMENTAL.

The experimental animals used were six male white rats (*Mus norvegicus albinus*) of about the same weight and age kept in a zinc-free environment. The cages for the individual rats were made entirely of copper rivetted together with copper rivets (7). After careful cleaning and drying they were heavily lacquered with a nitrocellulose lacquer and baked. With this treatment they successfully resisted wear, although small amounts of copper were occasionally dissolved. The feces and urines were collected separately in glass (7). The rats drank distilled water from burettes during the normal periods, and ammonium chloride and sodium bicarbonate solutions during the acid and alkaline periods, respectively. The food consumed was carefully weighed, and 100 gm. samples were repeatedly analyzed for calcium and zinc throughout the course of the investigation. An accurate account could be kept therefore of the zinc and calcium intake and

output. The routine data for each rat included measuring the volume of fluid intake, total volume of urine excreted, collection of feces, urine, and cage washings, weight of the animal, weight of food consumed, and finally analytical determinations of the calcium and zinc intake and of the output of these metals in urine and feces. In the case of each rat the results followed the general drift shown in Fig. 1 without exception, although there were of course minor fluctuations. Owing to the great number of individual determinations and to the uniformity of conditions throughout, the protocols and results for each individual animal have been omitted for the sake of brevity, and the intake and output of calcium and zinc in urine and feces considered for the entire group.

The diet selected had the following composition.

	<i>per cent</i>
• Cracked wheat.....	21.3
Corn-meal.....	21.3
Rolled oats.....	35.6
Milk powder (Klim).....	14.2
Liver ".....	4.7
Yeast ".....	2.8

This mixture was prepared in large quantity, carefully sampled for analysis, and samples occasionally drawn for analysis during the investigation. No zinc was added to the diet—the sole source of zinc was that naturally occurring in the food. In addition to the above ration, each rat was given a small weekly allowance of fresh lettuce to provide an adequate vitamin supply. The lettuce was weighed and samples retained for analysis, but the zinc and calcium were present in such minute amount that they did not enter as a factor in the salt balance. The rats thrived well on this diet and were clean and healthy at the termination of the experiment.

### *Methods of Analysis.*

The weekly output of urine and feces of each rat was separately ashed in Coors porcelain dishes. The urine, acid with HCl, was evaporated to dryness and the dry residue slowly ashed in an electric muffle furnace at a low temperature (8). The residue was extracted with 1:1 HCl and repeatedly ashed until all carbon had disappeared. The acid solution was analyzed for zinc by first

precipitating the iron from acid solution with cupferron, separating zinc as sulfide along with copper sulfide (a small amount of copper having been added to the original solution, in order to secure

TABLE I.  
*Summary of Metabolism Experiments.*

Period.	Type.	Urine.		Feces.	
		Zinc.	Calcium.	Zinc.	Calcium.
<i>wks.</i>		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	Water.	0.17	5.5	2.71	73.9
2	"	0.16	4.9	2.82	87.1
3	"	0.17	3.8	2.92	78.9
4	0.1 M NaHCO <sub>3</sub> .	0.34	4.3	3.67	78.4
5	0.1 " "	0.57	3.9	3.71	71.6
6	0.1 " "	0.52	4.9	3.14	97.0
7	0.1 " "	0.23	3.8	3.03	78.8
8	0.1 " "	0.16	3.8	3.03	79.2
9	0.1 " "	0.17	3.7	2.76	78.4
10	0.1 " "	0.23	3.6	2.67	72.3
11	Water.	0.17	2.7	2.68	82.0
12	1.0 M NH <sub>4</sub> Cl.	0.42	4.5	2.68	74.0
13	0.5 " "	0.40	12.6	2.23	61.9
14	0.4 " "	0.37	17.6	3.95	72.0
15	0.4 " "	0.39	19.7	3.56	78.4
16	0.4 " "	0.41	17.0	3.22	73.3
17	0.4 " "	0.46	19.9	2.67	68.1
18	0.4 " "	0.77	29.5	2.91	74.5
19	0.4 " "	0.98	26.1	3.11	71.3
20	0.4 " "	0.79	25.9	3.29	85.1
21	0.4 " "	0.66	23.5	3.43	74.2
22	0.4 " "	0.55	26.7	3.88	93.5
23	0.4 " "		8.4	2.88	105.0
24	0.4 " "	0.40	13.9	2.87	100.0
25	0.4 " "	0.47	11.2	3.03	112.0
26	0.4 " "	0.33	9.4	2.99	148.0

complete separation of zinc by entrainment) and, finally, separation of zinc from the copper. The estimation of zinc was made turbidimetrically in the case of zinc in urine, and volumetrically

in the case of zinc in feces by methods which have recently been described elsewhere (9). Calcium was determined in an aliquot

TABLE II.  
*Zinc Balance Experiments.*

Period.	Type.	Total Zinc.		Balance.
		Consumed.	Excreted.	
<i>wks.</i>		<i>mg.</i>	<i>mg.</i>	
1	Water.	3.23	2.88	+0.35
2	"	3.69	2.88	+0.81
3	"	3.79	3.09	+0.70
4	0.1M NaHCO <sub>3</sub>	3.66	4.01	-0.35
5	0.1 " "	3.75	4.28	-0.53
6	0.1 " "	3.78	3.66	+0.12
7	0.1 " "	3.67	3.26	+0.41
8	0.1 " "	3.83	3.19	+0.64
9	0.1 " "	3.78	2.93	+0.85
10	0.1 " "	3.30	2.90	+0.40
11	Water.	3.37	2.85	+0.52
12	1.0 M NH <sub>4</sub> Cl	3.33	3.10	+0.23
13	0.5 " "	2.22	2.43	-0.21
14	0.4 " "	3.12	4.32	-1.20
15	0.4 " "	3.06	3.95	-0.89
16	0.4 " "	2.43	3.63	-1.20
17	0.4 " "	2.59	3.13	-0.54
18	0.4 " "	2.73	3.68	-0.95
19	0.4 " "	2.76	4.09	-1.33
20	0.4 " "	3.33	4.08	-0.75
21	0.4 " "	2.96	4.09	-1.13
22	0.4 " "	3.24	4.43	-1.19
23	0.4 " "	3.06	(2.88)	
24	0.4 " "	3.07	3.27	-0.20
25	0.4 " "	3.47	3.50	-0.03
26	0.4 " "	3.48	3.32	+0.16

portion of the original solution of ash by McCrudden's volumetric method (10).

The food samples were similarly ashed and analyzed for calcium and zinc. In zinc analyses of this type in which such small

amounts of zinc were involved all necessary precautions were taken to avoid the accidental introduction of zinc during the course of the analysis. Only especially purified reagents were used (9).

TABLE III.  
*Calcium Balance Experiments.*

Period.	Type.	Total Calcium.		Balance.
		Consumed.	Excreted.	
<i>wks.</i>		<i>mg.</i>	<i>mg.</i>	
1	Water.	127.1	79.4	+47.7
2	"	145.2	92.0	+53.2
3	"	148.4	82.7	+65.7
4	0.1 M NaHCO <sub>3</sub> .	144.2	82.7	+61.5
5	0.1 " "	147.7	75.5	+72.2
6	0.1 " "	149.0	101.9	+47.1
7	0.1 " "	144.6	82.6	+62.0
8	0.1 " "	150.7	84.0	+66.7
9	0.1 " "	149.0	82.1	+66.9
10	0.1 " "	123.3	75.9	+47.4
11	Water.	133.0	84.7	+48.3
12	1.0 M NH <sub>4</sub> Cl.	131.2	78.5	+52.7
13	1.0 " "	85.9	74.5	+11.4
14	1.0 " "	122.7	89.6	+33.1
15	1.0 " "	119.5	88.1	+31.4
16	1.0 " "	95.5	90.3	+5.2
17	1.0 " "	102.0	95.5	+7.5
18	1.0 " "	107.4	104.0	+3.4
19	1.0 " "	108.5	97.4	+11.1
20	1.0 " "	131.2	111.0	+20.2
21	1.0 " "	116.7	97.7	+19.0
22	1.0 " "	127.7	120.2	+7.5
23	Water.	120.5	113.4	+7.1
24	"	121.0	113.9	+7.1
25	"	136.7	123.2	+13.5
26	"	198.4	157.4	+41.0

The technique of this method of analysis is not difficult to acquire, but more than ordinary precautions must be taken to guard against the accidental introduction of zinc during the course of

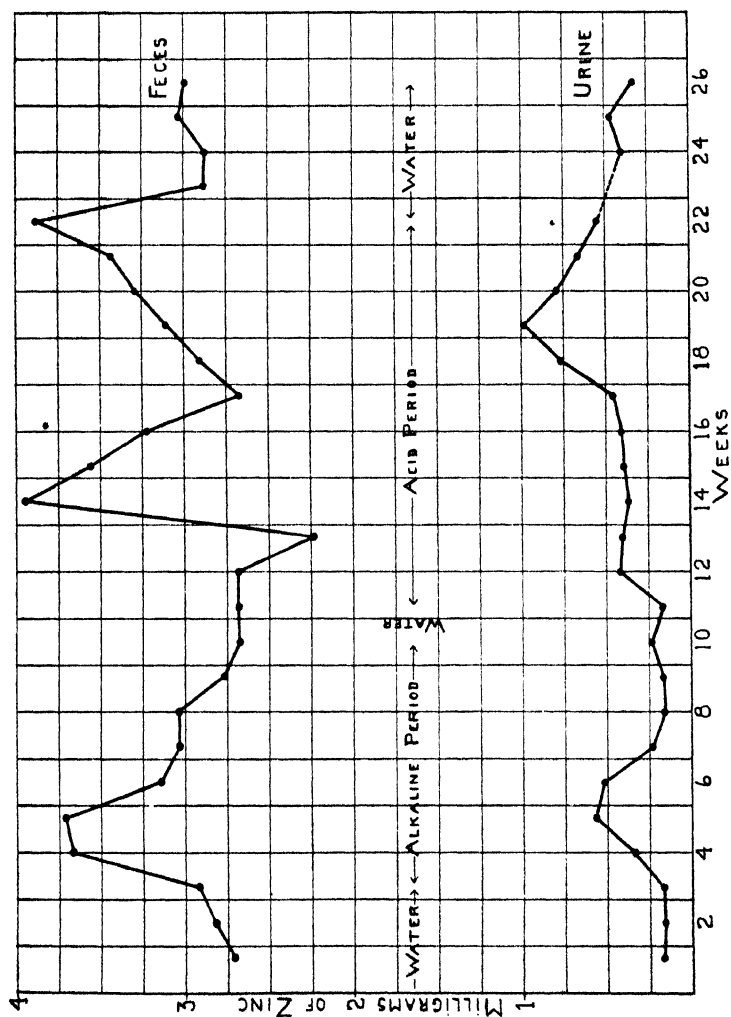


FIG. 1. Normal excretion of zinc by rats. Both urinary and fecal zinc output increase during the early part of the alkaline period but return to normal before the termination of the period. The output increases in both cases during the acid period.

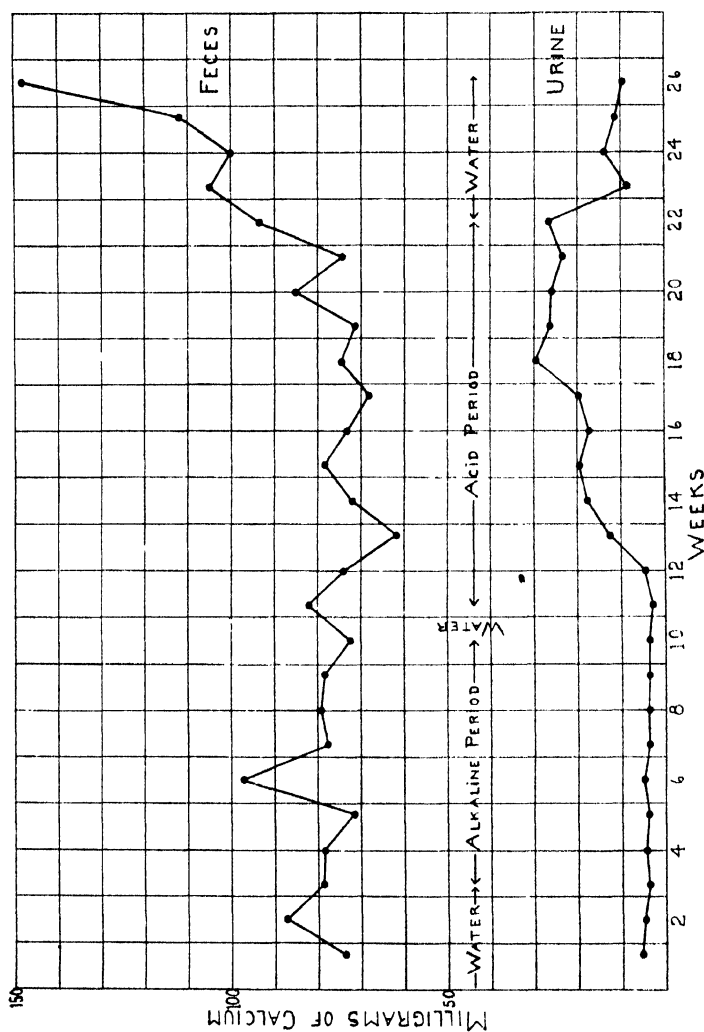


FIG. 2. Normal excretion of calcium by rats. The urinary excretion of calcium is unaffected by an alkaline diet but becomes decidedly increased during acid feeding. The fecal excretion is not greatly changed during either period. The rise during the final water period was due to increased food ingested.

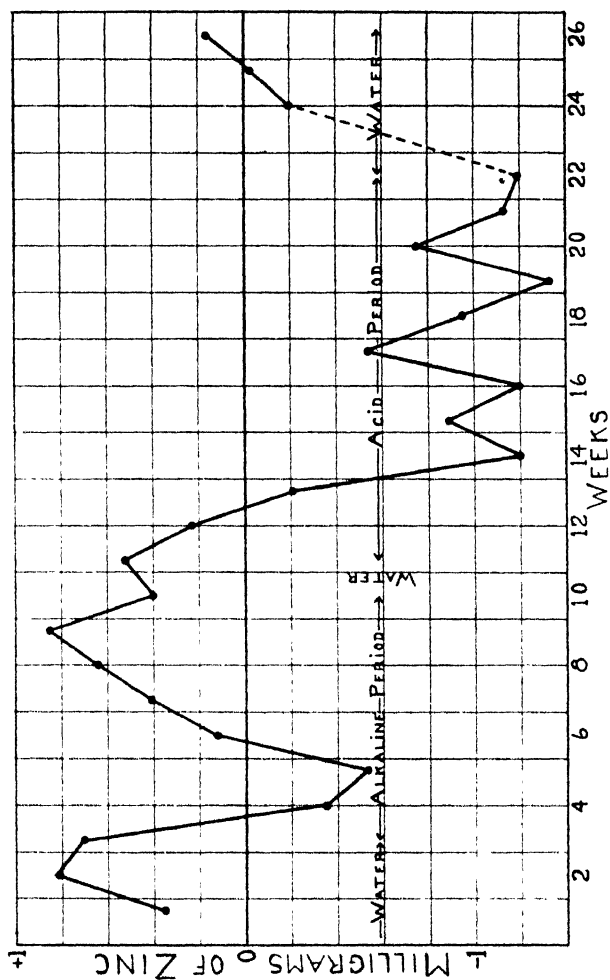


FIG. 3. Zinc metabolism experiments. The balance first becomes negative on an alkaline diet, but quickly returns to normal. On an acid diet the balance remains negative until the animal is returned to a normal diet.



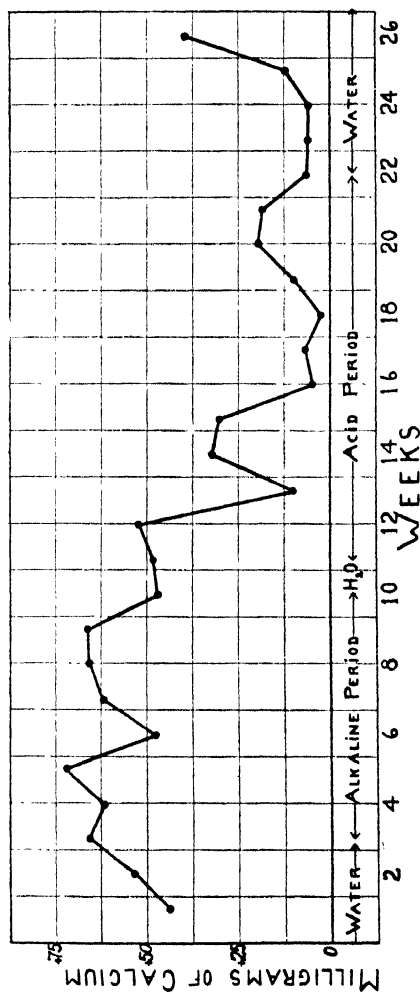


FIG. 4. Calcium metabolism experiments. Alkali is without perceptible effect upon the calcium balance. During the acid period the calcium balance drops but does not quite become negative.

the analysis or losses resulting from solubility effects of various reagents. The amount of zinc found in the urine (an average of 0.17 mg. per rat per week) was well within the range of the turbidimetric method, so that it is felt that confidence can be placed upon the accuracy of the results obtained, even though the amounts are small. These results have been gathered together in Tables I, II, and III and are presented graphically in Figs. 1 to 4.

#### DISCUSSION.

An analysis of the results obtained from these experiments reveals some interesting points. During both alkaline and acid periods of feeding there is a pronounced increased excretion of zinc, while the calcium excretion is appreciably affected only during the acid period. This is particularly apparent in the curves of urinary zinc and calcium excretion (Figs. 1 and 2). During the alkaline period the calcium output remains practically constant and of the same magnitude as that during the water periods. The zinc output, however, is decidedly affected and at first rises to a value three times as great as during the water period, after which it falls to normal before reaching the end of the alkaline period. There is evidently a readjustment of the organism to a condition of alkalosis which effectively prevents the loss of zinc. The mechanism of this readjustment is difficult to explain. The high initial output may be a consequence of the amphoteric character of zinc. Zinc hydroxide has acid properties as well as basic, whereas calcium is strongly basic only. Thus zinc hydroxide ionizes  $\text{Zn}(\text{OH})_2 \rightleftharpoons 2\text{H}^+ + \text{ZnO}^{--}_2$ , as well as  $\text{Zn}(\text{OH})_2 \rightleftharpoons \text{Zn}^{++} + 2\text{OH}^-$ , so that  $k = [\text{ZnO}^{--}_2] \times [\text{H}^+]^2$  has a value of  $0.71 \times 10^{-29}$ . It can act therefore as a weak acid and it is conceivable that this may be a factor in the initial increased excretion during alkalosis.

During the period of acid feeding the output of both zinc and calcium is greatly increased. Calcium is not sufficiently increased during this period to cause the balance to become negative, but the increased elimination is plainly apparent in both the fecal and urinary excretion curves and in the calcium balance chart. Zinc, on the other hand, becomes decidedly negative, both fecal and urinary zinc rising in amount during acid feeding. Zinc is, therefore, apparently more sensitive to changes in body condition than calcium.

The behavior of calcium noted above accords with observations made by Mendel and Givens (11). These investigators found that urinary calcium is strikingly increased during acid feeding. On the other hand sodium bicarbonate has no marked influence upon calcium metabolism. Mendel and Givens showed that the administration of large doses of sodium bicarbonate to a human diabetic does not increase the output of urinary calcium. They also demonstrated that the calcium balance is not affected by acid feeding, and further observations by Givens (12) have indicated that while urinary calcium output is increased following the ingestion of hydrochloric acid, it is without marked influence upon the total calcium metabolism. In the present experiments, however, both the fecal and urinary calcium output are distinctly increased, although the balance does not become negative. The rise in calcium output towards the end of the experiment (Fig. 2) is due to an increased food consumption following return to a normal period. The calcium balance (Fig. 4) at this point is not affected, of course, the values merely rising to normal. The zinc output does not drop to normal during the acid period, as during the alkaline period, but instead remains at a relatively high level until the animals are returned to a normal diet.

The average daily intake per rat during the water periods amounted to 0.48 mg. of zinc and 19.1 mg. of calcium. The average daily excretion per rat during these same periods amounted to 0.44 mg. of zinc and 15.1 mg. of calcium. There is, therefore, a small constant storage of both zinc and calcium which the animal derives from its normal diet. Since the ratio  $\text{Ca}:\text{Zn} = 4.0:0.04$ , or 100:1, the zinc storage at this period amounted to 1 per cent of the calcium storage. The rate of calcium storage is of about the same order as that found by Sherman and MacLeod (13) for adult rats.

The storage of zinc at the rate of 0.04 mg. per day as found during this period is probably not fixed, since the zinc metabolism is readily affected by conditions of acidity and alkalinity. Besides it varies with age, being greater with young animals and less as the age increases. Table IV and Fig. 5 illustrate the rapid manner in which zinc is accumulated in the organism of a growing rat. These analyses indicate the zinc and calcium content of the rat minus the hide. The gastrointestinal tract was washed out with distilled

water in all cases except that of the very young rat. While the concentration of zinc is not appreciably changed during this period of growth, the rate of accumulation is greater than later on. The total amount of zinc in the animal varies from 0.27 to 1.03 per cent of the total calcium, as may be seen from the calcium:zinc ratios. It is interesting to observe that the relation between zinc and calcium is of the same order (nearly 1 per cent) as that maintained in storage, but distinctly lower than the relative values existing in the blood stream.

The ratio of calcium to zinc in the blood stream is about 14:1 and figures taken from the literature substantiate this in general, but since no analyses exist for calcium and zinc in the blood of the same rat, the following analyses were carried out for the purpose

TABLE IV.  
*Total Calcium and Zinc Content of Rats of Various Ages.*

Age of rats.	Calcium.	Zinc.	Ca : Zn.
<i>days</i>	<i>gm.</i>	<i>mg.</i>	
7	0.069	0.37	186 : 1
21	0.235	2.33	101 : 1
35	0.389	4.00	97 : 1
70	1.223	6.25	195 : 1
112	1.605	7.67	209 : 1
210	3.016	8.20	368 : 1

of obtaining data upon this point. Two large adult male rats were chloroformed and immediately bled. The blood samples were ashed and total calcium and total zinc determined in each. The results are summarized in Table V.

The zinc content of the blood of rats as shown in this table amounts to about 7 per cent of the calcium content. It would be interesting to observe in this connection whether zinc follows the same variation or is related to the change in calcium content of blood which follows the administration of parathyroid to normal animals (14).

There is this difference in salt utilization between calcium and zinc; namely, zinc is somewhat less firmly retained. A shift in the condition of the body to either an acid or an alkaline condition is quickly reflected in losses of zinc, whereas calcium excretion is

not affected by an alkaline condition and there is somewhat of a lag in excretion during the acid period. Furthermore, the average proportionate increase in calcium excretion during the acid period compared with that during the preliminary water periods,

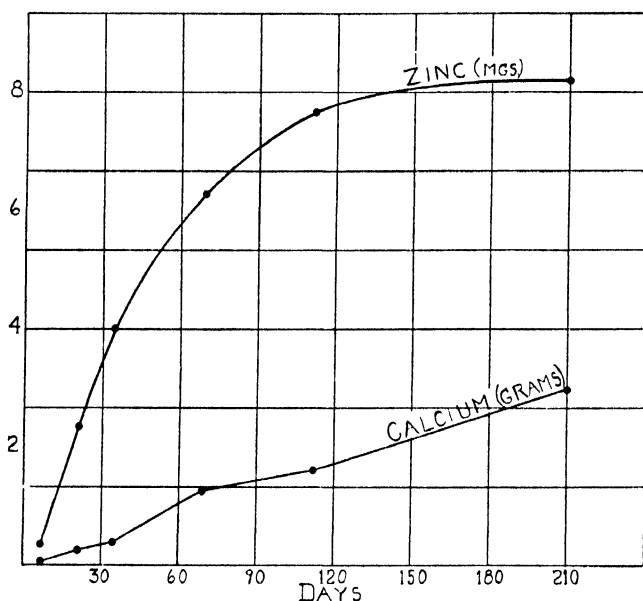


FIG. 5. Rate of zinc and calcium storage in growing rats. Zinc is more rapidly stored in the growing animal than in the adult.

TABLE V.  
*Calcium: Zinc Ratio in Rat Blood.*

Rat No.	Calcium, <i>mg. per 100 gm.</i>	Zinc, <i>mg. per 100 gm.</i>	Ca: Zn.
1	10.2	0.68	15 : 1
2	10.4	0.79	13 : 1

$\frac{95.1 - 84.6 \times 100}{84.6} = 12.4$  per cent, is much less than that for zinc during the same periods,  $\frac{3.73 - 2.92 \times 100}{2.92} = 27.7$  per cent.

One can only speculate as to the importance of zinc to the living

animal. The relatively large amount present in the blood stream points to some important function. A man of 72 kilos in weight possesses some 6 kilos of blood and has, therefore, about 30 mg. of zinc constantly circulating through his tissues, in addition to the zinc which is an integral part of these tissues. Various tentative suggestions have been put forth that zinc may serve as a

TABLE VI.  
*Normal Zinc Content of Various Foodstuffs.*

Raw foodstuffs.	Zinc. mg. per kg.
Peanut butter.....	20.6
Shredded wheat.....	40.5
Bread.....	4.1
Graham crackers.....	13.0
Corn-meal.....	8.8
Wheat flour.....	8.0
Rolled oats.....	40.0
Liver (dried).....	117.4
Butter.....	2.5
Oranges.....	1.1
Grapenuts.....	13.9
Spinach.....	3.1
<hr/>	
Preserved in glass.	
Spinach.....	12.3
Green beans.....	4.9
Tomatoes.....	5.9
Beets.....	57.8
Plum jam.....	5.7
Marmalade.....	10.2
Peaches.....	3.8

catalyst for enzymic activity and its relation to growth has recently been emphasized (15); still no definite function has yet been found.

*Zinc a Factor in the Salt Metabolism of Man.*

The wide distribution of zinc in foodstuffs and particularly its concentration in germ cells, the endosperm of wheat, yolk of eggs, etc., point to zinc as an inorganic substance which is especially essential during periods of early growth; its constant presence and

amount in the blood and tissues of adult animals point to a definite utilization. Thus, a man consumes from 10 to 15 mg. of zinc per day in his food.

As an illustration of the quantities of zinc present naturally in various foodstuffs, Table VI is appended of analyses which were made to secure control data for nutrition experiments.

Except in the case of liver, which was dried and powdered, the above analyses refer to the zinc content per kilo as purchased.

TABLE VII.  
*Fecal Zinc Excretion of a Man on a Normal Diet.*

Period.	Total zinc.
<i>days</i>	<i>mg</i>
1	6.75
2	8.75
3	9.25
4	5.00
5	5.50
6	8.25
7	5.00
8	10.50
9	19.70
10	69.80
11	36.30
12	18.23
13	11.20
14	7.58
15	6.98
16	17.10
17	11.87
18	9.56
19	14.62

Man consumes in this way 10 to 15 mg. of zinc daily. His urinary excretion averages 1 mg. per day and does not fluctuate within wide limits, while his fecal excretion varies considerably depending upon the amount of zinc ingested (Table VII). Fig. 6 represents this daily fecal excretion of zinc by a man over a period of nearly 3 weeks. An average mixed diet was consumed and only varied in that on 1 day (the 9th) the food was of a type particularly rich in zinc. The immediate increase in excretion following this is plainly indicated in Fig. 6.

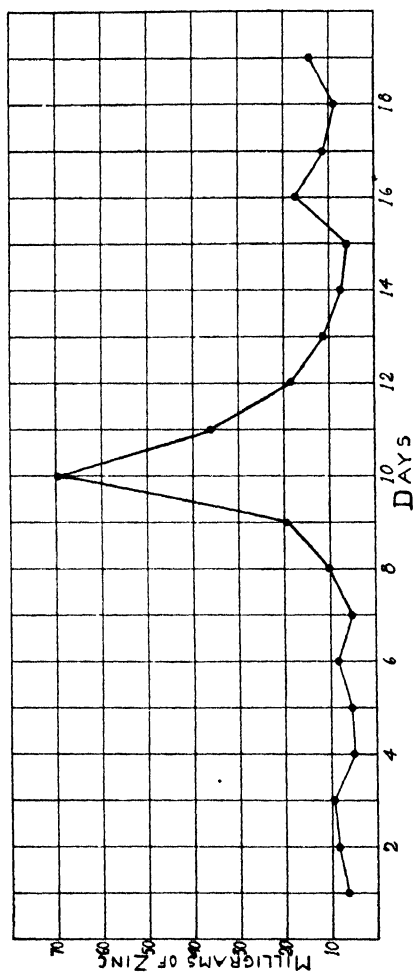


FIG. 6. Normal fecal excretion of zinc by a man on an average mixed diet. On the 9th day a plate of oysters was consumed and followed by a considerable increase in zinc excretion.



It is apparent from these results that man ingests and absorbs daily a significant amount of zinc. His tissues are continually bathed in a medium which contains zinc and in addition he carries a large store of zinc in his tissues (15). It would be of interest to observe how the zinc metabolism of man varies in health and disease, a subject as yet unexplored.

#### CONCLUSIONS.

The zinc metabolism of rats has been investigated and compared with their calcium metabolism under various conditions. Under conditions of alkaline feeding, both urinary and fecal zinc excretion are at first increased and the balance becomes negative, followed by a return to normal. Similarly, under an acid régime both urinary and fecal zinc excretion are increased, the balance becomes negative, but remains so until the animals are returned to a normal diet.

The calcium metabolism is not affected by an alkaline diet, for both fecal and urinary calcium excretion remain normal during this period, whereas, with an acid diet, both the fecal and urinary calcium excretion are increased somewhat, although the balance does not become negative.

The utilization of zinc by the rat is about 1 per cent and the amount circulating in the blood stream is about 7 per cent of the calcium involved in each case. The rate of storage of zinc, as is the case with calcium, is greatest in the young growing animal.

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## BLOOD CHANGES IN ACUTE MERCURIC CHLORIDE POISONING.

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Very little information is available as to the blood changes occurring in acute mercurial poisoning especially over short periods of time. Myers and Fine<sup>1</sup> report a case but they do not give any figures for the first 5 days and only at intervals of 2 to 3 days thereafter. In the following paper there is given the results of an investigation on a fatal case of bichloride poisoning. The blood samples were taken every 8 hours beginning 30 hours after the poison was swallowed and continuing until death ensued 10 days later.

The patient was a young girl about 18 years of age and weighed 140 pounds. Only one 7.5 gr. tablet was taken at 6.30 p.m., February 7, 1925. Medical assistance was summoned immediately and within 20 minutes, milk and egg whites were given, and the stomach washed out with sodium bicarbonate solution. Fluids were forced and milk of magnesia, bismuth, and egg white given every hour.

Treatment with sodium thiosulfate and glucose intravenously was instituted and continued until death. 25 cc. of a 4 per cent solution of sodium thiosulfate in normal saline, and 10 per cent glucose in normal sodium citrate in amounts varying from 1500 to 400 cc. were given every 8 hours.

The poison was very quickly absorbed and kidney function ceased about 5 hours after the poison was taken. 120 cc. of urine were passed at 10.05 p.m., 40 cc. at 11.15 p.m., and 23 cc. at 8.30 a.m. the next morning. For 5 days there was complete anuria and on the 6th day 90 cc. of urine were voided. On the 8th and 9th days 240 and 150 cc. of urine respectively were obtained by catheter. The patient died on the morning of the 10th day.

The blood pressure was 95/62 and the white blood count was 55,000 on the 2nd day and 67,000 on the 3rd day.

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<sup>1</sup> Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xx, 391.

TABLE I.

Time. (1)	Non- protein nitrogen. (2)	Urea nitrogen.		Amino acid nitrogen.		Uric acid. (7)	Uric acid nitrogen.		Remarks. (10)
		(3)	(4)	(5)	(6)		(8)	(9)	
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent N.-P. N.</i>	<i>mg. per 100 cc.</i>	<i>per cent N.-P. N.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent N.-P. N.</i>	
Feb. 7, 6.30 p.m.									One 7.5 HgCl <sub>2</sub> tablet swallowed. Voided 120 cc. urine. " 40 " " " 23 " " Anuria.
10.05 "									
11.15 "									
Feb. 8, 8.30 a.m.									
Feb. 9, 12.00 "	66.5	Urease method failed.		8.54	12.84	6.67	2.22	3.34	
9.00 "	83.5	62.5	74.9	10.50	12.56	6.90	2.30	2.75	"
Feb. 10, 12.30 "	105.0	65.8	62.7	9.03	8.60	7.10	2.37	2.26	"
8.30 "	112.5	71.4	63.4	8.82	7.84	9.00	3.00	2.67	"
Feb. 11, 12.00 "	107.5	71.4	66.4	9.94	9.25	9.50	3.17	2.95	"
8.00 "	112.5	76.2	67.8	8.68	7.72	9.70	3.23	2.87	"
4.00 p.m.	120.0	75.7	63.1	8.82	7.35	10.10	3.37	2.81	"
Feb. 12, 12.00 a.m.	120.0	85.0	70.8	7.14	5.95	10.30	3.43	2.85	"
8.00 "	115.0	85.6	74.4	6.94	5.60	10.00	3.33	2.89	"
4.00 p.m.	125.0	87.0	69.6	7.14	5.71	10.10	3.37	2.70	"
Feb. 13, 12.00 a.m.	122.5	77.6	73.4	7.70	6.39	8.50	2.83	2.31	"
8.00 "	118.0	86.2	72.6	7.14	6.01	9.20	3.07	2.57	"
10.00 "									Voided 90 cc. urine.
Feb. 14, 12.00 "	155.0	67.6	43.6	7.49	4.83	8.50	2.83	1.83	Anuria.
8.00 "	153.5	72.9	47.6	7.28	4.76	8.80	2.93	1.91	"
4.00 p.m.	169.0	82.2	48.7	9.17	5.43	9.00	3.00	1.78	"

Feb. 15, 12.00 a.m.	176.0	98.8	51.2	9.31	5.29	10.20	3.40	1.93	"
8.00 "	188.0	109.0	58.0	8.40	4.47	10.00	3.33	1.76	"
4.00 p.m.	180.0	108.0	60.0			10.70	3.55	1.98	Catheterized specimen, 240 cc.
Feb. 16, 12.00 a.m.	184.0	120.0	65.2	9.87	5.36	12.50	4.17	2.27	
8.00 "	208.0	118.4	56.9	10.22	5.84	12.40	4.13	1.98	
4.00 p.m.	234.0	136.6	58.4	14.00	5.98	15.60	5.20	2.22	Catheterized specimen, 150 cc.
Feb. 17, 12.00 a.m.	192.0	143.8	74.5	12.32	6.42	16.50	5.50	2.86	
9.00 "	214.0	135.2	63.0	12.74	5.95	16.70	5.57	2.49	Died 10.56 a.m.

The specimens of vomitus which were saved yielded about 3 gr. of mercuric chloride, and traces were found in the urine. As not all of the vomitus was obtained it appears that between 4.0 and 4.5 gr. of mercury were absorbed.

The methods employed were those given in Folin's system of blood analysis. The urea was determined after hydrolysis in the autoclave as the first determination which was attempted by the urease method failed because of the poisoning of the catalase by the mercury.

The figures for the total non-protein nitrogen are given in Column 2 of Table I. It will be noted that there is a steady rise in the non-protein nitrogen until February 14, when there occurs a marked increase. This increase follows the taking of the first food after the poisoning and is accompanied by change for the worse in the patient's condition. Up to this time the patient was bright and mentally alert but from this time on she was in coma much of the time and appeared much weaker, and when aroused, complained of her vision being blurred and her arms and legs being numb.

As can be seen in the next two columns of Table I, there is at this time a marked drop in the urea as a result of the kidneys again starting to function. In spite of this drop in the urea the patient for the first time became comatose. It would seem that the liver and other urea-forming organs had lost much of their power and that as a result of the patient's partaking of food there had been a great increase in the nitrogenous products other than urea and amino acids. It would also seem probable that as a result of the injury to the intestinal mucosa the absorption of these toxic products was markedly increased. In Table II it will be seen that there is a tremendous increase in the amount of undetermined nitrogen. As expressed in terms of non-protein nitrogen this factor has been varying between 15 and 20 per cent. It now jumps suddenly to over 40 per cent and then drops again as the amount of urea increases.

The amino acid figures are given in Columns 5 and 6 of Table I. As might be expected there is no appreciable increase in amino acid until about the last 24 hours before death. In terms of the non-protein nitrogen there is a progressive fall from the high figure of 12.84 per cent to the low one of 4.47 per cent 2 days before death.

The uric acid figures are given in Columns 7, 8, and 9 of Table I. It will be seen that the uric acid has not increased in proportion to the other nitrogenous constituents. This agrees with the observations of Folin, Berglund, and Derick<sup>2</sup> that man is able to destroy from 10 to 70 per cent of the uric acid injected intravenously. In several cases they report figures of over 18.0 mg.

TABLE II.

Time.	Undetermined nitrogen.		Blood sugar.	Remarks.
	mg. per 100 cc.	per cent N.-P.N.	mg. per 100 cc.	
Feb. 9, 12.00 a.m.			71	Anuria.
9.00 "	2.47	2.96	101	"
Feb. 10, 12.30 "	21.60	20.59		
8.30 "	23.06	20.50	119	"
Feb. 11, 12.00 "	16.65	15.48	78	"
8.00 "	17.14	15.23	88	"
4.00 p.m.	25.00	20.81	105	"
Feb. 12, 12.00 a.m.	17.45	14.54	119	"
8.00 "	12.37	10.77	112	"
4.00 p.m.	19.74	15.79	112	"
Feb. 13, 12.00 a.m.	14.20	11.58	98	"
8.00 "	14.69	12.35	98	"
10.25 "				90 cc. urine.
Feb. 14, 12.00 "	68.90	44.51	116	
8.00 "	62.20	40.50	116	
4.00 p.m.	66.10	39.14	98	
Feb. 15, 12.00 a.m.	62.80	35.63	111	
8.00 "	56.70	30.19	109	
4.00 p.m.			142	240 cc. urine.
Feb. 16, 12.00 a.m.	38.00	20.67	154	
8.00 "	60.04	29.05	139	
4.00 p.m.	64.00	27.34	140	150 cc. urine.
Feb. 17, 12.00 a.m.	16.73	8.66	140	
9.00 "	44.60	20.83	147	

per 100 cc. of plasma, while the highest figure given here is only 16.7 mg. per 100 cc. of whole blood.

In Table III the figures for creatinine and creatine are recorded. The creatinine is found to increase steadily while the creatine remains fairly constant. With the increase in non-protein nitrogen the creatine percentage falls consistently until February 14,

<sup>2</sup> Folin, O., Berglund, H., and Derick, C., *J. Biol. Chem.*, 1924, lx, 361.

when the patient became comatose. During this period of stupor the amount of creatine increased much more rapidly than the creatinine. It would appear that the conditions which were responsible for the coma also caused an alteration in the creatinine and creatine metabolism. In the case of Myers and Fine the

TABLE III.

Time.	Creatinine.	Creatinine N.		Total creatinine.	Total creatine N.		Creatine N.	
	mg. per 100 cc.	mg. per 100 cc.	per cent N.-P.N.	mg. per 100 cc.	mg. per 100 cc.	per cent N.-P.N.	mg. per 100 cc.	per cent N.-P.N.
Feb. 9, 12.00 a.m.	6.72	2.16	3.25	14.7	5.47	8.22	3.31	4.97
9.00 "	7.44	2.39	2.86	15.4	5.73	6.86	3.34	3.95
Feb. 10, 12.30 "	8.22	2.68	2.55	16.5	6.14	5.85	3.46	3.29
8.30 "	8.94	2.87	2.55	16.9	6.29	5.59	3.42	3.04
Feb. 11, 12.00 "	10.56	3.39	3.15	17.1	6.36	5.92	2.97	2.76
8.00 "	11.7	3.75	3.33	19.3	7.18	6.38	3.43	3.05
4.00 p.m.	12.8	4.11	3.42	19.1	7.11	5.93	2.99	2.49
Feb. 12, 12.00 a.m.	12.8	4.11	3.42	18.9	7.03	5.86	2.92	2.43
8.00 "	13.2	4.24	3.69	19.6	7.29	6.34	3.05	2.65
4.00 p.m.	14.0	4.49	3.59	20.8	7.74	6.20	3.25	2.60
Feb. 13, 12.00 a.m.	14.2	4.56	3.72	20.8	7.74	6.32	3.18	2.59
8.00 "	14.4	4.62	3.89	20.7	7.70	6.48	3.08	2.59
10.25 "								
Feb. 14, 12.00 "	14.4	4.62	2.98	21.8	8.11	5.23	3.49	2.25
8.00 "	14.8	4.75	3.11	21.5	8.00	5.23	3.25	2.13
4.00 p.m.	16.4	5.26	3.11	22.5	8.37	4.95	3.11	1.84
Feb. 15, 12.00 a.m.	16.4	5.26	3.05	28.2	10.49	5.95	5.23	3.03
8.00 "	16.6	5.33	2.87	28.2	10.49	5.58	5.16	3.01
4.00 p.m.	16.8	5.40	3.00	30.6	11.39	6.33	5.99	3.36
Feb. 16, 12.00 a.m.	18.6	5.97	3.29	32.1	11.95	6.50	5.98	3.25
8.00 "	19.2	6.16	2.96	34.8	12.95	6.23	6.79	3.26
4.00 p.m.	18.2	5.84	2.49	38.1	14.18	6.06	8.34	3.57
Feb. 17, 12.00 a.m.	18.0	5.78	3.01	39.0	14.52	7.56	8.74	4.55
9.00 "	18.6	5.97	2.79	44.4	16.55	7.73	10.58	4.94

the same phenomena are noted. Their figures show that at first the creatinine exceeded the creatine but that later when the patient developed symptoms of uremia the creatine exceeded the creatinine.

Their figures for all the constituents except uric acid are much higher than those given in this case. At the end of 5 days during which both cases showed anuria they report a non-protein nitrogen

of 258 mg. and a urea nitrogen of 183 mg., while I find only 125 mg. of non-protein nitrogen and 87 mg. of urea nitrogen. In spite of the fact that their patient voided 2989 cc. of urine in the next 5 days in contrast to only 480 cc. for mine, the non-protein nitrogen in their case increased from 258 mg. to 338 mg. while that in mine reached a maximum of only 234 mg.

Even with the much lower nitrogen figures shown in my case coma and death ensued on the 10th day, while their patient lived for 15 days more and did not develop uremia until the 18th day, at which time the non-protein nitrogen and urea nitrogen were at the same level as on the 10th day.

The figures for the blood sugar are given in Table II. There is no appreciable increase in the blood sugar levels until the last 2 days, despite the fact that the patient was receiving from 150 to 240 gm. of glucose each day. It would appear that mercury does not injure the power of the cells to utilize glucose.

#### SUMMARY.

The blood changes resulting from poisoning from 4.0 to 4.5 gr. of mercuric chloride have been followed every 8 hours. It is noted that during the period of coma the undetermined nitrogen showed a very marked increase, and that the amount of creatine exceeded that of creatinine. This finding agrees with that shown in the case reported by Myers and Fine.

The amino acid nitrogen and the uric acid did not increase in proportion to the rise in the other nitrogenous constituents. The failure of the uric acid to show a greater increase confirms the findings of Folin, Berglund, and Derick as to the destruction of uric acid in the body.





## PENTOSE METABOLISM.

### I. THE DISPOSAL OF INTRAVENOUSLY ADMINISTERED XYLOSE IN THE RABBIT.\*

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For a number of reasons, considerable interest attaches itself to metabolism of the sugars having five carbon atoms. They are employed in building up various tissues of the body; they are excreted in the urine under conditions of unknown etiology; they occur in varying amounts in the dietary of all animals and yield a certain amount of energy. However, the balance of evidence is undoubtedly that the pentoses of the tissues are not from those of the diet, the latter possibly contributing to the unfermented reducing substances of normal urine. Idiopathic pentosuria, as well, seems independent of the food, possibly being of glycolytic origin. In the form of the pentosans, the pentoses comprise a large proportion of the diet of the herbivora, and part of the diet of other animals in so far as they consume vegetable products. There is evidence that the pentoses may be burned or stored as glycogen in the animal body, at least in small amounts, the utilization of the pentosans of course being dependent upon the extent to which they are broken down in the alimentary tract. Further study of the metabolism of the pentoses should be of interest not only for itself but also because there might be obtained information in regard to more general tissue reactions and particularly to the metabolism of the hexoses.

The determination of the variations in the concentration of various substances in the blood has been of inestimable value in the study of a great many of the problems of metabolism. Such

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a study of the pentoses is handicapped by the lack of methods of analysis.

The very similar chemical properties, correlated with closely corresponding structural configurations of the pentoses and hexoses, makes difficult the elaboration of chemical methods for the determination of one in the presence of the other. It is possible, nevertheless, to employ more or less satisfactory biological means of separation. The resistance of pentoses to fermentation by ordinary baker's yeast has furnished a way that has been used at times to separate the two types of substances for analytical purposes. This is the basis of the method that has been employed to determine the pentoses present in the blood, following their administration.

Such a method is open to a number of obvious objections. The fermentation itself may introduce errors, since the yeast may contain reducing substances, may form reducing substances during fermentation, may fail to destroy completely the glucose of the blood, or may change the reducing properties of the substances other than glucose in the blood (1). However, since under similar conditions the reducing power of the blood after fermentation with yeast is small and fairly constant, no real difficulty arises. It is scarcely unjustifiable to assume that in the presence of pentoses the difference from the usual unfermented reducing power of the blood furnishes a reliable index of the amount of pentoses present. In the absence of direct evidence, there is no reason for assuming that the presence of the pentoses would cause any significant change in the usual unfermented reduction.

The present communication reports the effect on blood sugar concentrations, following the intravenous administration of xylose to the rabbit under varying conditions. The concentration of any substance in the blood is dependent upon the interrelationships between the processes of addition, destruction, storage, and excretion, the relative importance of each of which can frequently be determined by experimental alterations. In regard to xylose, the addition can be absolutely controlled, the excretion changed to a certain extent, while the rates of destruction and storage are much more difficult to alter. While the primary excretory channel for unassimilable sugars may be said to be the

kidneys, some if not all of the foreign sugars when present are excreted partly into the intestine, from where they may be re-absorbed and the process repeated. Undoubtedly bacterial action may destroy part of these sugars while in the intestine.

TABLE I.  
*Control Experiment.\**

Rabbit 2, 2.2 kilos; black male.

Date.	Time.	Blood.			Urine sugar
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.	
1925		mg.	mg.	mg.	gm.
Oct. 26.....	9.50 a.m.	45.0	128.0	27.0	0
" 26.....	10.50 "		136.0	25.4	0
" 26.....	11.45 "		133.6	15.6	0
" 26.....	1.45 p.m.		126.8	15.6	0

\* Two experiments performed.

TABLE II.  
*Effect of the Administration of Glucose.\**

Rabbit 3, 1.8 kilos; tan male.

Date.	Time.	Blood.			Urine sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1925		mg.	mg.	mg.	gm.	
Oct. 19	9.20 a.m.	50.6	90.2	21.4		1 gm. of glucose at 9.30 a.m.
" 19	9.35 "		326.1	21.4		
" 19	10.35 "		111.0	25.4		
" 19	11.35 "		145.0	14.0		
" 19	1.30 p.m.		142.0	15.6	0	

\* Two experiments performed.

These facts complicate the interpretation of the results of the administration of sugars either intravascularly or enterally. Undoubtedly, they would tend to invalidate absorption experiments that are not very carefully controlled, since otherwise there would be obtained the differential rather than the actual absorp-

tion. Since renal excretion must be the eventual fate of those unassimilable sugars that are not lost by way of the alimentary tract either by bacterial destruction or failure of reabsorption, in

TABLE III.

*Effect of Xylose on the Blood Sugar.\**

Rabbit 4, 2.0 kilos; tan male.

Date.	Time.	Blood.			Urine, Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
		mg.	mg.	mg.	mg.	
1925						
Oct. 28	9.15 a.m.	43.2	98.4	11.0		1 gm. of xylose at 9.30 a.m.
" 28	9.35 "		254.4	110.0		
" 28	10.35 "		146.6	56.4		
" 28	11.35 "		136.0	35.8		
" 28	1.35 p.m.		136.0	11.0	485	Urine from 9.15 a.m. to 1.35 p.m.
" 30	1.35 "				206	

\* Five experiments performed.

TABLE IV.

*Effect of Tartrate Nephritis on the Blood Sugar.\**

Rabbit 7, 2.4 kilos; tan male.

Date.	Time.	Blood.			Urine. Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1925		mg.	mg.	mg.	mg.	
Nov. 30	9.30 a.m.	40.2	98.4	19.2		1.5 gm. of tartaric acid in molecular $\text{Na}_2\text{CO}_3$ at 10 a.m., Nov. 30.
Dec. 1	9.30 "	112.0	114.0	28.1		
" 1	5.30 p.m.	149.0	75.2	38.2	10	
" 2	9.30 a.m.	203.8	156.8	47.6	20	

\* Two experiments performed.

this study, there have been induced tartrate nephritis and phlorhizin diabetes as types of decreased and increased kidney permeability, respectively.

Since the liver is undoubtedly of great importance in at least certain phases of carbohydrate metabolism, disordered liver func-

TABLE V.

*Effect of Tartrate Nephritis on the Blood Sugar with the Administration of Xylose.\**

Rabbit 2, 2.4 kilos; black male.

Date.	Time.	Blood.			Urine, Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1925		mg.	mg.	mg.	mg.	
Nov. 30	10.00 a.m.	46.4	156.8	38.1		1.5 gm. of tartaric acid in molecular solution of $\text{Na}_2\text{CO}_3$ at 10.15 a.m.
Dec. 1	9 00 "	116.0	119.2	40.0		1 gm. of xylose at 9.15 a.m.
" 1	9 20 "		254.8	154.2		
" 1	10 20 "		207.0	103.6		
" 1	11 20 "		161.8	82.8		
" 1	1 20 p.m.		135.6	60.0		
" 1	5 20 "	139.7	90.4	43.8	5	
" 2	9 30 a.m.	186.7	147.6	52.4	2	

\* Five experiments performed.

TABLE VI.

*Effect of Chloroform Poisoning on the Blood Sugar.\**

Rabbit 5, 1.8 kilos; tan male.

Date.	Time.	Blood.			Urine sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1925		mg.	mg.	mg.	mg.	
Dec. 7	3.30 p.m.	38.9	118.0	26.6	0	1 cc. of chloroform in 4 cc. of olive oil at 4 p.m.
" 8	2.00 "	60.4	89.0	28.2	0	
" 9	2.00 "	127.7	99.8	38.2	0	

\* One experiment performed.

tion resulting from chloroform poisoning has been studied to determine the effect, if any, on the disposal of the injected xylose.

The relationship between sugars having five and six carbon atoms as probably evidenced by the origin of the anabolic pentoses and of those of the pentosurias, has made it appear of interest to determine any possible correlation between the fate of xylose and insulin, which latter is of such importance in at least certain phases of carbohydrate metabolism.

There have also been conducted several preliminary experiments in regard to the effect of enterally introduced xylose, and

TABLE VII.

*Effect of Chloroform Poisoning on the Blood Sugar with the Administration of Xylose.\**

Rabbit 10, 2.4 kilos; brown male.

Date.	Time.	Blood.			Urine. Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1926		mg.	mg.	mg.	mg.	
Jan. 4	10.00 a.m.	43.7	98.4	17.2		1 cc. of chloroform in 4 cc. of olive oil at 10.10 a.m.
" 5	9.05 "	60.8	63.8	29.8		1 gm. of xylose at 9.15 a.m.
" 5	9.25 "		293.2	193.6		
" 5	9.55 "		161.8	103.6		
" 5	10.25 "		151.6	79.0		
" 5	11.25 "		126.8	52.4		
" 5	2.30 p.m.	96.3	119.2	31.4	300	
" 5	5.00 "			23.0		

\* Four experiments performed.

one in regard to the temporary disposal of the xylose that is not immediately excreted.

#### EXPERIMENTAL.

The details of this series of experiments may be summarized as follows: 1 gm. of xylose in 15 cc. of water was injected into the marginal ear vein of a rabbit that had been fasting for 24 hours. The blood samples (2 to 3 cc.) were collected at intervals using potassium oxalate as an anticoagulant, and analyzed for non-protein nitrogen, blood sugar, and reducing power after fermentation. The proteins were precipitated and the non-

protein nitrogen determined according to the Folin-Wu procedure, and the reducing power of the filtrate determined by the Shaffer-

TABLE VIII.

*Effect of Phlorhizin on the Blood Sugar.\**

Rabbit 13, 1.5 kilos; tan male.

Date.	Time.	Blood.			Urine. Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1928		mg.	mg.	mg.	mg.	
Jan. 18	10.45 a.m.	52.6	85.4	13.8		0.3 gm. of phlorhizin at 11 a.m.
" 19	11.00 "	56.9	97.0	9.0	0	Much sugar.
" 20	11.00 "	59.2	107.8	4.4		
" 21	11.00 "	59.2	114.0	4.4	0	

\* One experiment performed.

TABLE IX.

*Effect of Phlorhizin on the Blood Sugar, with the Administration of Xylose.\**

Rabbit 15, 2.0 kilos; white male.

Date.	Time.	Blood.			Urine. Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1928		mg.	mg.	mg.	mg.	
Feb. 22	10.00 a.m.	34.2	101.4	17.2		0.7 gm. of phlorhizin at 10.15 a.m.
" 23	10.10 "		98.4	17.2		1.0 gm. of xylose at 10.20 a.m.
" 23	10.25 "		228.6	139.2		
" 23	11.25 "		121.6	55.6		
" 23	12.40 "		101.2	21.0	339	Total urine sugar 454 mg.
" 23	1.30 p.m.		109.0	17.2	123	" " " 237 "
" 23	2.40 "	33.8	103.6	13.8	269	" " " 424 "

\* Three experiments performed.

Hartmann method, the results being expressed in terms of glucose. The xylose employed had 96 per cent of the reducing power



of glucose, which corresponds with results that have been reported by others (2).

*Fermentation of Blood Sugar.*—A number of preliminary experiments on goat blood proved satisfactory using a suspension of Fleischmann's yeast in water. When, however, rabbit blood was tried very anomalous results were obtained, the reducing power of the blood increasing with the time of fermentation. A possible explanation may be that a high content of amylase of the blood of the rabbits used attacked the starch contained in the yeast cakes. Since it is possible to remove this starch by centrifuga-

TABLE X.

*Effect of Insulin on the Blood Sugar with the Administration of Xylose.\**  
Rabbit 19, 2.9 kilos; tan male.

Date.	Time.	Blood.			Urine, Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
		mg.	mg.	mg.	mg.	
1926						
Mar. 10	9.15 a.m.	37.2	111.4	13.8		1 gm. of xylose and 2 units of insulin at 9.50 a.m.
" 10	10.00 "		220.2	116.6		
" 10	11.00 "		92.0	48.2		
" 10	12.00 "		114.0	28.2	325.6	
" 10	1.00 p.m.		121.6	13.8		
" 10	2.00 "		119.2	9.0		
" 10	3.00 "		116.6	13.8	142.6	

\* Two experiments performed.

tion, the starch suspension used in these experiments was prepared as follows: A suspension of one-half cake of Fleischmann's compressed yeast in 20 cc. of 0.9 per cent sodium chloride solution was centrifuged in a conical tube giving a separation into a lower white layer of starch and an upper gray layer of yeast. The supernatant liquid was poured off and the yeast was mixed with 10 cc. of the isotonic salt solution. 1 cc. of whole blood mixed with 0.5 cc. of the yeast suspension was incubated an hour at 37°C. The proteins were removed by the Folin-Wu method and the filtrate analyzed for residual reduction. 5.0 mg. of glucose per cc. were removed completely, while 1.0 mg. of xylose per

cc. was destroyed only slightly (5 per cent). Variation of the time of incubation within a range of from 30 to 90 minutes had scarcely any effect. Sodium fluoride was found to interfere slightly with the fermentation.

The use of the Shaffer-Hartmann method for low concentrations of sugar has been criticized by a number of investigators (3, 4). Duggan and Scott (4) have stated that there is a lack of oxidizing power of the reagent when very low concentrations of the reducing substances are present. They report, however, that when reduction does take place at the low concentrations the results are consistent, although they would substitute figures of

TABLE XI.

*Effect of Enterally Administered Xylose on the Blood Sugar.\**

Rabbit 22, 2.9 kilos; tan male.

Date.	Time.	Blood.			Urine. Unfermented sugar.	Remarks.
		Non-protein nitrogen.	Total sugar.	Unfermented sugar.		
1926		mg.	mg.	mg.	mg.	
Mar. 2	9.25 a.m.	43.2	106.4	21.0		3.0 gm. of xylose by stomach tube at 9.35 a.m.
" 2	10.35 "		161.8	68.8		
" 2	11.10 "		151.6	66.2		
" 2	11.55 "		139.2	58.6	614	
" 2	1.35 p.m.		126.8	28.2		
" 2	3.35 "		119.2	13.8	300	

\* Three experiments performed.

their own for those originally published by Shaffer and Hartmann. The results of these experiments are calculated from the table of the latter. Since the interest lies in the relative rather than the absolute values, no attempt has been made to check over the figures at these low concentrations. It has been found, however, that consistent results are obtained from the analysis of the same solution.

*Fermentation of Glucose in the Urine.*—The pentoses in the urine (*i.e.* the unfermented reduction) were determined by the Shaffer-Hartmann micro method after fermentation with a yeast suspension for 1 hour at 37°C. The urine was so diluted that 5 cc.

would contain an amount of sugar that was within the limits of the reagent. 20 cc. of this diluted urine after fermentation with 2 cc. of the yeast suspension were centrifuged and the supernatant liquid analyzed. 5 to 8 per cent of xylose in urine or aqueous solution was destroyed under these conditions.

### *Results.*

Typical examples of each kind of experiment are given in Tables I to XI. Tables I, II, and VIII indicate no marked variations in residual reduction of the blood in the normal animal, in the normal animal after the administration of glucose, or in an animal poisoned with phlorhizin. Tartrate nephritis (Table IV) and chloroform poisoning (Table VI) are associated with an increased residual reduction which is possibly due in large measure to the accompanying increase in the non-protein nitrogen of the blood (3). Subsequent to the administration of xylose, the unfermented reducing power of the blood returned to its previous value in 4 hours in the normal animal (Table III) and in chloroform poisoning (Table VII), 2 hours in phlorhizin diabetes (Table IX), but was scarcely back to the control level in 8 hours in tartrate nephritis (Table V). The simultaneous intravenous administration of xylose and insulin gave an effect not strikingly different from that of xylose alone. When 3 gm. of xylose were given by stomach tube there resulted a rise in residual reduction less marked but of longer duration than that resulting from the intravenous injection of one-third as much. Except after the administration of glucose, and of insulin, the total sugar of the blood roughly paralleled the unfermented fraction.

### DISCUSSION.

Although, as often reported, a large portion of administered xylose may be recovered in the urine, it is not possible to account for it all in this manner. Since quantitatively renal elimination is one of the important methods of pentose disposal, it is not unexpected that phlorhizin diabetes and tartrate nephritis, representing decided differences in kidney permeability, should cause a marked increase and decrease respectively in the rate of removal of the xylose from the blood and its excretion in the urine.

Renal elimination is not the only mechanism involved, however,

since, as exemplified in Table III, the residual reduction of the blood was definitely lowered to the usual value and yet the urine for the next 48 hours contained 206 mg. of unfermented sugar. Assuming that even as much as 10 per cent of 2 kilos, the weight of the animal, was blood, 200 cc. could not possibly have held a fraction of this amount without detection. It is of interest to observe that by such a method of calculation, which though crude should give figures of significance, it is not possible to account for all the administered xylose immediately after the injection.

Undoubtedly, all the methods of removal contribute to this result. Probably oxidation plays a rôle, but a relatively unimportant one, since so much of the sugar can be recovered in the urine. While it seems not unlikely that intestinal excretion may take place, with subsequent reabsorption, in consideration of the extended time during which pentose remains in the urine, it would appear that this is not the only other fate.

Thus far, the only experiment to determine the method of disposal of xylose was the same as that of Table III, except that the animal was killed at the end of 4 hours, when the residual reduction of the blood had returned to its previous level. The kidneys, liver, intestinal contents, and the muscles of one hind leg were boiled 30 minutes with enough water to cover, acidified with acetic acid, and filtered. Of the protein-free extracts, that of the liver alone gave a qualitative reduction test, 40 mg. of unfermented sugar (?) being found. No conclusions can be drawn from one experiment with no controls, but it is of interest to note that the alimentary tract apparently did not contain the pentose that was unaccounted for in the urine (383 mg. found).

Folin and Berglund (5) have interpreted some of their results as indicating that non-assimilable carbohydrates or carbohydrate derivatives are stored and gradually eliminated. They showed that "dextrin" (probably unassimilable derivatives) continued to be excreted even though the alimentary tract was thoroughly cleaned out. Xylose is apparently subjected to similar treatment in part, but the nature of the storage is obscure, further experimentation being necessary to decide whether it is as such or otherwise. While polymerization would seem rather unlikely, it is well to continue to consider it until definitely disproven (6).

The results of the experiments with chloroform poisoning might

possibly be taken as showing the absence of any important relationship between the disposal of xylose and the functions of the liver, particularly as regards glucose metabolism in any of its ramifications. As yet, however, such a conclusion is rendered hazardous, in consideration of the absence of any exact criterion of the extent of liver destruction. Furthermore, it is well to keep in mind the belief expressed by Delprat and Whipple (7), "that the liver has a very large factor of safety—that it can tolerate extensive injury and yet carry on its essential body functions." A significant sugar tolerance test, such as one using fructose, is rather objectionable because of its possible interference with the effect of xylose.

As administered, insulin was without marked effect, possibly because of a lack of relationship between ordinary carbohydrate metabolism and that of xylose. Voegtlin, Dunn, and Thompson (8) have reported that xylose as well as arabinose has only a questionably antagonistic action on insulin intoxication.

In conclusion, it is admitted freely that the greatest of caution should be observed in interpreting the results of any acute experimentally produced abnormality. In most cases, undoubtedly more than one type of tissue or function is affected to some extent, and furthermore in case of injury of one organ, it may be possible for another to assume its duties at least in part. Thus Nash and Benedict (9) suggest the view, "that phlorhizin not only affects the permeability of the kidney tissue to blood sugar, but produces an intrinsic impairment of the sugar-burning mechanism."

#### SUMMARY.

1. Subsequent to the administration of xylose, the reducing power of the blood after fermentation returned to its previous value in 4 hours in the normal rabbit, 2 hours in phlorhizin diabetes, 4 hours in chloroform poisoning, but was scarcely back to the control level in 8 hours in tartrate nephritis.

2. Urinary excretion is an important factor in the disposal of intravenously administered xylose.

3. The evidence would seem to indicate that part of the intravenously administered xylose is stored and gradually excreted in the urine.

4. No evidence was obtained of any important relationship between ordinary carbohydrate metabolism and that of xylose.

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## NITROGEN METABOLISM IN THE CHICK EMBRYO.

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To determine the nature of the food material burned by the developing chick embryo use has been made of two methods in the past. The validity of the information to be derived from one of them—the measurement of the respiratory quotient—we shall presently have occasion to examine. The second and more reliable method is based upon direct analysis of the egg contents at various stages of development; by means of it the fact has long since been established that most of the organic matter oxidized during the period of incubation must be fat.<sup>1</sup>

This method of direct analysis has been used most effectively by Murray.<sup>2</sup> He has recently determined the content of both fat and total solids in eggs which had been incubated for 16 to 19 days. Basing his calculations on the average composition of unincubated eggs, he estimates that the loss in total solids during the development of the embryo is equal, within the experimental error, to the loss in fat. The experimental error however is considerable, and Murray is willing to concede that fat may represent no more than 90 per cent of the total solids that have disappeared. He argues then that protein may be as much as 10 per cent of all the substance burned, but cannot possibly be more.

In reaching this conclusion Murray has overlooked one factor which is by no means negligible; namely, the weight of the nitrogenous waste products. Later in this paper we shall show that the chief end-product of protein metabolism in the chick embryo, as in adults of the same species, is uric acid. In its nitrogen content, 1 gm. of protein is equivalent to about 0.5 gm. of uric acid.

<sup>1</sup> Needham, J., *Physiol. Rev.*, 1925, v, 1.

<sup>2</sup> Murray, H. A., Jr., *J. Gen. Physiol.*, 1925-26, ix, 1, 39.



Hence the diminution in total solids incident to the combustion of a given quantity of protein will amount to only about half the weight of the protein that is burned. If it is true, then, that 10 per cent of the loss in dry matter during incubation is a consequence of protein combustion, the ratio of protein to total substance oxidized must be not 10, but nearly 20, per cent.

On the other hand the observations made by Murray, as well as those of earlier investigators,<sup>1</sup> are not definitely inconsistent with the view that no protein is destroyed at all. If this should be the case, the chick embryo must possess the unusual capacity of living on fat as the sole source of energy, at least after the 10th or 11th day of incubation—for by that time the 0.2 gm. or so of sugar which the egg originally contained has disappeared.<sup>3</sup> The embryo is moreover growing rapidly, hence there is also the interesting possibility that this organism can transfer protein from the white and yolk to its own tissues without waste. When an infant is capable of utilizing 50 per cent of its protein intake for purposes of growth, the performance is considered quite remarkable.<sup>5</sup> If the chick embryo can utilize 100 per cent, as it must if no protein is burned, the fact would be of no little general interest.

Ostensibly there is one bit of evidence tending to show that the embryo's protein consumption is quite negligible, but it is based upon a misconception. We refer to the experiments of Hasselbalch<sup>4</sup> on gas metabolism during incubation. He found that, from the 5th day on, the respiratory quotient is in the neighborhood of 0.68, a figure which is low even for pure fat and would ordinarily be considered to rule out protein combustion. Hasselbalch however was in error in supposing that the quotient uni-

<sup>3</sup> Gadaskin, I. D., *Biochem. Z.*, 1926, clxxii, 447 (for earlier literature see Needham<sup>1</sup>). 0.2 gm. is merely a rough estimate. All the published data are in per cent of sugar in the yolk and white. By the 5th day, according to Gadaskin, the sugar is half gone. Hence, if the total quantity in the unincubated egg is 0.2 gm., 0.1 gm. has disappeared within the first 5 days. The combustion of 0.1 gm. of sugar would yield 75 cc. of carbon dioxide, whereas within this period the egg gives off no more than 10 cc. The remainder of the sugar disappears between the 5th and the 11th days, but after the 5th day the respiratory quotient is regularly low.<sup>4</sup> Probably, therefore, most of the sugar is not burned, but has some other fate.

<sup>4</sup> Hasselbalch, K. A., *Skand. Arch. Physiol.*, 1900, x, 353.

<sup>5</sup> Cf. Rubner, M., and Langstein, L., *Arch. Physiol.*, 1915, 39.

versally ascribed to protein in dealing with mammalian organisms (0.80) is applicable to the chick embryo as well. When, as here, the principal end-product is uric acid rather than urea, the respiratory quotient for protein is practically identical with that for fat. It follows that the gas exchange during embryonic life in birds provides no information whatsoever on the extent to which protein is burned. The calculation given below is based upon the assumption that egg albumin is the sole source of protein and that all the nitrogen excreted is in the form of uric acid.

100 gm. albumin = 53.0 gm. C, 7.10 gm. H, 22.0 gm. O, 15.9 gm. N.

47.7 gm. uric acid + 9.46 gm. water = 17.0 gm. C, 2.20 gm. H, 22.0 gm. O, 15.9 gm. N.

Leaving to be burned. 36.0 gm. C, 4.90 gm. H.

The oxidation of this residue requires 94.4 liters of oxygen and yields 67.3 liters of carbon dioxide. The respiratory quotient is  $67.3 \div 94.4$ , or 0.71.

Somewhere between a negligible fraction and 20 per cent of the total solid matter oxidized by the chick embryo is therefore protein. Analysis for fat and total solids only will evidently not suffice for a more accurate estimate, and measurements of gas exchange are of no use. More precise knowledge on this point probably cannot be obtained except by the method which has furnished substantially all the information we possess on the rate of protein combustion in other organisms; namely, the analysis of urine. The results of our attempts in this direction will form the chief subject of this paper.

The very meagre literature on excretion by the embryonic kidney at first sight appears to be discouraging to any such investigation. Not until the period of incubation has more than half expired does the metanephros attain its full development. The mesonephros, which alone in the first 11 days or more of embryonic life is in a position—speaking anatomically—to dispose of the special waste products of protein metabolism, is stated to be functionless in this regard. That water passes through the mesonephros even against pressure is proved experimentally by the fact that hydronephrosis follows obstruction of the Wolffian duct.<sup>6</sup> The anatomical “evidence” that this organ is otherwise functionally inert seems to be merely an expression of opinion,<sup>6</sup>

<sup>6</sup> Boyden, E. A., *J. Exp. Zool.*, 1924, xl, 437.

and the evidence founded on previous analyses of the embryonic fluids is no more conclusive. Analysis of the allantoic fluid by earlier investigators has been confined to a few physical measurements: (1) the freezing point determinations of Bialaczewicz,<sup>7</sup> which show that the osmotic pressure of this fluid is about equal to that of the blood of adult vertebrates, and (2) the observations of Aggazzotti<sup>8</sup> on the hydrogen ion concentration, revealing the slight degree of alkalinity characteristic of blood generally. The latter author has convinced himself that the fluid delivered into the allantoic cavity by the mesonephros, inasmuch as it is not acid in reaction, cannot be a true excretion. Evidently he has been misled by the fact that the yolk and white together are in the ordinary sense an "acid" food and would, when eaten by an adult animal, produce an acid urine. But in the embryo synthetic processes are going on at an extraordinary rate, relatively speaking. Whether acid or base will predominate in the small residuum which the embryo finally rejects no one could possibly predict. Furthermore, if the allantoic wall is permeable to electrolytes, an excretion originally acid would not necessarily remain so, for the establishment of equilibrium with the fluid contents of contiguous structures would tend to make the pH approximately the same throughout.

Nothing short of chemical analysis of the allantoic fluid for possible waste products could decide whether the mesonephros is an active excretory organ, and until now such analyses have not been made. We have been able, in the course of this investigation, to demonstrate a measurable amount of uric acid in the allantoic fluid almost as soon as connection with the Wolffian duct has been established, and as development proceeds a steady rise in the concentration of this substance.

Chemical analysis of the allantoic fluid was suggested to us in the first place by certain toxic manifestations which appear when the normal progress of Wolffian duct development is prevented, as reported by Boyden<sup>6</sup> in another place. While the suspended growth of the allantois in such experimentally altered embryos might be explained entirely on mechanical grounds, histological changes in its wall (edema, diapedesis) point to the presence of some other factor, presumably the accumulation of waste products.

<sup>7</sup> Bialaczewicz, K., *Arch. Entwicklungsmech. Organ.*, 1912, xxxiv, 489.

<sup>8</sup> Aggazzotti, A., *Arch. ital. biol.*, 1913, lix, 305.

The hypothesis that metabolism products are retained when the Wolffian duct has been obstructed carries with it the assumption that the mesonephros has an excretory function involving other substances than water, and is entirely out of line with the alleged inutility of this structure. We therefore looked for uric acid in the allantoic fluid and, having found it, undertook a preliminary survey of its concentration at various stages of development. The work eventually grew into a more or less complete metabolism study—as far as the main nitrogenous waste products are concerned—covering more especially the first 2 weeks of embryonic life. Partly because the amniotic cavity has been said to serve, during the last half of incubation, as a repository for products of excretion,<sup>8</sup> and partly because of the intimate contact between the amnion and the allantoic sac, we have made some observations also on the amniotic fluid.

The correlation of our analytical results with the structural development of the chick will be undertaken in a separate publication.<sup>9</sup> The purpose of this paper is to present the strictly chemical side.

### *Methods.*

The eggs were obtained from White Leghorn hens during the months from April to July, 1924, and from March to June, 1925. As soon as received they were placed in a Petaluma incubator maintained at a temperature of  $39.5^{\circ} \pm 1.0^{\circ}$ . The humidity was regulated roughly by keeping a dish of water in the incubator, and the eggs were turned and cooled twice a day.

*Collection of Allantoic and Amniotic Fluids.*—Until the end of the 5th day of incubation the allantois is a minute bag, a few mm. in diameter, which can without great difficulty be isolated from surrounding structures and ligated at its neck. In the earliest specimens, accordingly, the sac was tied off in that manner and cut away beyond the ligature. The contents were then collected in a test-tube by puncturing the allantoic wall, and the sac itself with its adhering fluid transferred to another tube containing water. Later, when the fluid was analyzed, accurately measured samples were withdrawn with dry pipettes, and what remained was added to the washings. Uric acid was determined in both the undiluted fluid and the washings, and the two results then added to give the total quantity.

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<sup>9</sup> Boyden, E. A., and Fiske, C. H., to be published.

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By this means we were able also to calculate the original volume of the fluid, and the determination of the concentration of any other substance in the undiluted portion gave us all the information that we needed in order to ascertain its total quantity. The washings could not have been used for all the analyses required, for they contained material (partly protein) extracted from the allantoic wall, etc. On this account direct determinations of the total nitrogen content of the washings would not have been dependable. An example of the sort of calculation which permitted us to overcome this difficulty is given below.

Age, 5 days. Number of embryos, 12. Volume of undiluted fluid (A) used for analyses, 4.00 cc. The rest of the undiluted fluid (less than 1 cc.) together with the washings (about 6 cc.) was made up to a volume of 50 cc. (B).

### Analyses:

Concentration of total nitrogen in undiluted fluid (A).....	30.7	mg. per cent.
Concentration of uric acid in undiluted fluid (A).....	16.4	" " "
Concentration of uric acid in B.....	0.334	" " "

### Calculations:

Total amount of uric acid in A (4 cc.).....	$\frac{4}{100} \times 16.4$	= 0.656	mg.
Total amount of uric acid in B (50 cc.).....	$\frac{50}{100} \times 0.334$	= 0.167	"
Sum.....		0.823	"
Uric acid per embryo.....	$0.823 \div 12$	= 0.0694	"
Volume of allantoic fluid per embryo.....	$\frac{0.0694}{16.4} \times 100$	= 0.423	cc.
Total nitrogen per embryo.....	$\frac{0.423}{100} \times 30.7$	= 0.130	mg.

In the case of embryos more than 5 days old, removal of the allantoic sac *in toto* is not feasible. Here the allantois was approached through the air space, the shell membrane peeled away, and the fluid sucked out as thoroughly as possible with a fine pipette. Hemorrhage was stopped with cotton. To recover the fluid still remaining in the allantoic cavity, the latter was washed *in situ* with 15 to 50 cc. of water, the amount depending on the age. Finally the cavity was drained by partially inverting the entire egg and cautiously rotating it about its long axis.

In the later stages, urate is found deposited in the form of solid masses. These were recovered with the aid of forceps, and analyzed separately as described below.

The amniotic fluid was collected with a fine pipette, no attempt at quantitative recovery being made, as only the concentration was of interest.

*Weighing.*—Following the collection of material for analysis, the embryo

was transferred to normal saline solution, where the membranes were cut off at the level of the vitelline duct. The embryo was then weighed rapidly on a watch glass, after removing the surplus salt solution as completely as it could be done with a capillary pipette. Thorough drying of the surface of the embryo by means of filter paper or any such absorbent substance results in loss of fluid from the body cavity, hence the slight error caused by liquid adhering to the epidermis must be accepted.

The embryonic weight was used to detect gross abnormalities in development. The various samples of allantoic fluid collected at one sitting were kept separate until the weights were known. Whenever an embryo proved to be much heavier or lighter than the average for the set, the fluid which had been obtained from it was discarded. The remaining samples were then mixed together, and the analyses begun.

The embryos of our series, probably because of the higher incubation temperature, were regularly about 35 per cent heavier than Murray's. The growth equation which he gives<sup>2</sup> is  $\log W = 3.6 \log t - 0.175$ , where  $W$  is the weight in mg. and  $t$  the time in days. The equation which most closely fits our data is  $\log W = 3.45 \log t + 0.104$ . If the slope is assumed to be identical with that of Murray's curve (3.6), the best line that can be drawn through our experimental points is  $\log W = 3.6 \log t - 0.043 = 3.6 \log \frac{t}{0.92} - 0.175$ .

This indicates that our embryos, as compared with Murray's, reached any given stage of their development in about 8 per cent less time.

*Analytical Methods.*—Total nitrogen and uric acid were the only constituents determined systematically, the former by the method of Folin and Denis,<sup>10</sup> the latter by various modifications of the colorimetric method, as outlined below. Occasionally the allantoic fluid was analyzed for creatine and for amino acids, using the methods of Folin.<sup>11, 12</sup>

No one method of estimating uric acid is suitable under all conditions. As a routine measure we used the direct colorimetric procedure in its most recent form.<sup>13</sup> In uncontaminated allantoic fluid, uric acid appears to be the only substance giving any color on heating with pure<sup>14</sup> phospho-18-tungstic acid when cyanide alone is used to make the mixture alkaline. Previous precipitation with zinc chloride and sodium carbonate,<sup>15</sup> or with ammoniacal silver magnesia mixture, does not lower the result (Table I). On the other hand, protein-free filtrates prepared from the tissues of the embryo itself contain a large amount of chromogenic matter which is precipitated by silver lactate, but cannot be extracted from the silver precipi-

<sup>10</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

<sup>11</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

<sup>12</sup> Folin, O., *J. Biol. Chem.*, 1922, li, 393.

<sup>13</sup> Folin, O., *J. Biol. Chem.*, 1922, liv, 153; Laboratory manual of biological chemistry, New York and London, 4th edition, 1925, 247.

<sup>14</sup> Folin, O., and Trimble, H., *J. Biol. Chem.*, 1924, lx, 473.

<sup>15</sup> Morris, J. L., *J. Biol. Chem.*, 1919, xxxvii, 231.

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TABLE I  
*Comparison of Methods.*  
*Uric Acid Concentration in Undiluted Allantoic Fluid.*

Age of embryo.	Direct method.	Precipitation with zinc chloride and sodium carbonate (Morris).	Precipitation with ammoniacal silver magnesia mixture.
<i>days</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
5	12.1*	12.0	
7	52.0		51.6
11	151		149
12	172	172	
13	239	242	235

*Uric Acid in Washings from One Embryo.†*

	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
6	0.141‡	0.145	
8	1.05‡	1.10	
	1.06§		
11	2.06		2.00
12	4.14	4.12	
13	9.88	9.93	

\* Another sample of this fluid was analyzed by precipitating with silver lactate, the uric acid being extracted with NaCl-HCl solution before the color was developed. The result was 11.8 mg. per 100 cc. At later stages, silver precipitation is incomplete, just as in the case of pure urate solutions.

† As stated elsewhere, the washings were ordinarily so dilute that the uric acid could not be precipitated from them quantitatively. In order to test the accuracy of the direct method as applied to such material we were therefore obliged to collect special samples for the purpose, using much less water for rinsing out the allantoic cavity.

‡ Trace of protein precipitated with uric acid reagent (see text).

§ 10 cc. of washings were mixed with 0.1 cc. of horse serum, and the protein removed by heat coagulation in the presence of a mixture of sodium acetate and acetic acid. Two filtrations of the resulting suspension, which had meanwhile been made up to a volume of 25 cc., gave a perfectly clear solution. When an aliquot of this was analyzed by the direct colorimetric method, no turbidity appeared. This experiment was done as a check on the heat coagulation method, which we used in the few instances where the washings contained more than a mere trace of protein.

tate with an acid solution of sodium chloride. In spite of this, the washings of the allantoic cavity, which contain a small amount of material extracted from the surrounding tissues, can also be analyzed without the use of a precipitating agent for the uric acid (Table I). This fortunate fact permitted us to use a large amount of wash water, thereby avoiding any risk of incomplete recovery of the allantoic fluid. In many instances the washings were so dilute that quantitative precipitation of the uric acid could not have been accomplished by any method known. The reliability of the direct colorimetric method in this case, combined with its extraordinary delicacy, has made it a very simple matter to estimate the volume of the fluid and the total output of each waste product, as illustrated by the sample calculation shown above. We doubt that any other satisfactory way of doing this could have been found.

In the early stages of this work the analysis of the washings gave some trouble, for a little protein is likely to be present. The removal of this protein, which interferes in the uric acid method, is easily accomplished by heat coagulation except when the contamination is very slight, and then it cannot be coagulated. There are doubtless many ways of getting rid of a small trace of protein, but none that we have tried is equal in convenience to precipitation with the uric acid reagent itself. The supernatant fluid, after centrifuging, when heated with cyanide in the usual way, then gives a clear blue solution which can be matched against the standard perfectly. A standard containing formalin cannot be used when, as in this case, the uric acid reagent is added before the cyanide.<sup>16</sup> The constant preparation of fresh uric acid standards has nevertheless been unnecessary, for a recently analyzed sample of protein-free allantoic fluid has always been on hand, and makes a wholly satisfactory standard.

The solid masses of urate—which may be found in the allantois as early as the 12th day, and towards the end of the incubation period completely fill the allantoic cavity—call for special treatment. They are coated with mucin-like material, and are less readily soluble in lithium carbonate solutions (and other mildly alkaline reagents) than is pure uric acid. The only way in which we have succeeded in dissolving these deposits is to let them stand for several hours moistened with a little strong hydrochloric acid. We have generally allowed most of the hydrochloric acid to evaporate, then nearly neutralized the remainder, and finally brought the uric acid into solution with the aid of a warm phosphate mixture.<sup>17</sup>

### *Results.*

#### *Concentration of Uric Acid and Other Nitrogenous Products in the Allantoic Fluid.*

The concentration of uric acid (or any other nitrogenous constituent) in the allantoic fluid is by itself of minor consequence

<sup>16</sup> Folin, O., *Laboratory manual of biological chemistry*, New York and London, 4th edition, 1925, 249.

<sup>17</sup> Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.



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from the standpoint of metabolism, and yet in some respects such information is of value. At the very outset (*i.e.* before the end of the 5th day) the composition of this fluid, with regard to its crystalloid nitrogenous constituents (Table II), corresponds approximately with what is found in the plasma of adults.<sup>18</sup> At this stage, then, there may be some doubt that the allantoic fluid represents a true excretion. Until about the end of the 5th day—when the uric acid concentration begins to rise with some rapidity—it may be no more than a mere filtrate. On the other hand

TABLE II.  
*Concentration of Uric Acid, Etc., in Allantoic Fluid.\**

Age.	No. of samples	Uric acid.	Total nitrogen.	Residual nitrogen.†
<i>days</i>		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
4.5	1	6.2	30.3	28.2
4.6	1	7.6		
4.8	3	4.9-11.5	24.5-26.7	21.2-22.9
5	5	11.9-16.4	24.2-30.7	20.0-25.6
6	5	19.5-41.1	24.6-29.8	15.1-18.9
7	5	32.0-50.6	21.7-31.9	8.7-16.7
8	5	60-111	35.9-48.8	11.8-18.3
9	4	61-140	33.3-69.0	10.8-22.3
10	5	80-140	44.4-68.0	12.1-23.4
11	3	99-179	51.3-74.6	14.9-20.5
12	4	128-252	64.9-86.4	10.3-29.1
13	5	203-360	103-113	24.8-42.7
14	3	235-533	129-216	38.5-50.6

\* Turbid fluids were cleared by warming before taking samples for analysis.

† Total nitrogen minus uric acid nitrogen.

some possibility exists that nitrogen excretion is well under way before the mechanism for uric acid synthesis has been developed. The exact time at which the active excretion of waste products begins is hence an open question, but the process is surely in full operation during the last part of the 5th day.

Inasmuch as uric acid is the only specific substance which we have determined regularly, we shall use the term "residual ni-

<sup>18</sup> Bock, J. C., *J. Biol. Chem.*, 1917, xxix, 191. Folin, O., Berglund, H., and Derick, C., *J. Biol. Chem.*, 1924, lx, 361.

trogen" here to include all nitrogenous products with the exception of this one. In the very early stages the residual nitrogen—thus defined—is by far the larger fraction, representing 85 per cent or more of the total nitrogen.<sup>10</sup> The concentration of the residual nitrogen later on diminishes, only to rise again—though slowly, and by no means in proportion to the rise in uric acid—from the 7th day on.

Somewhat earlier than the 3rd week of incubation the allantoic fluid has acquired more uric acid than it will dissolve (as urate). Sometimes as early as the 7th day the fluid is milky in appearance; on the other hand it is frequently quite clear as late as the 13th day, although the uric acid concentration then exceeds 0.2 per cent. The turbidity, when present, disappears on warming, so there is never any difficulty in getting representative samples of the fluid for analysis.

Urate deposits of a different nature have appeared however by the 13th day, and may even be seen in traces when only 9 days have elapsed. These deposits—already mentioned briefly in discussing the technique—are at first minute, soft, stringy masses, coated with protective material of some sort, and warming has no effect upon them. At the 11th day or later, they have begun to coalesce into brittle, flattened plaques. Gradually, as time goes on, more and more of the urate present in the allantoic cavity is deposited in this form. By the 19th day, at least as much as 87 per cent of it may be solid urate, while the amount left in solution may be no more than would be found on the 7th or 8th day.

One other physical characteristic worth mention is the yellow color, which has been observed before.<sup>8</sup> In our experience the allantoic fluid always has a yellowish tinge after the 11th day of incubation, while before that time it is invariably colorless.

<sup>10</sup> About half the residual nitrogen at this time, as will be seen below, is made up of amino acids. From our experience in determining the total nitrogen in 5 day allantoic fluids they must contain a not inconsiderable amount of non-nitrogenous organic matter, for they have proved to be quite remarkably resistant to digestion. At this stage the heating time prescribed for protein-free blood filtrates<sup>11</sup> is never adequate to bring about complete decolorization of 0.5 cc. of allantoic fluid, containing about the quantity of nitrogen found in 5 cc. of normal blood filtrate. By the 7th day, and sometimes earlier, this difficulty has disappeared, the refractory material by that time having been diluted 5 or 10 times by urine excreted through the mesonephros.

TABLE III.

*Total Quantity of Uric Acid, Etc., in Allantoic Fluid per Embryo.*

Sample No.	Age.	No. of embryos.	Volume.	Uric acid.	Total nitrogen.	Residual nitrogen.
	<i>days</i>		<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	4.5	20	0.11	0.007	0.034	0.032
2	4.8	18	0.21	0.025	0.053	0.045
3	4.8	20	0.30	0.034	0.082	0.071
4	5.1	10	0.24	0.032	0.061	0.050
5*	5.1	12	0.26	0.040		
6	5.0	15	0.35	0.050	0.107	0.090
7	5.1	14	0.47	0.077	0.120	0.094
8*	5.0	12	0.42	0.069	0.129	0.106
9	6.0	7	0.74	0.182	0.200	0.139
10	6.1	6	0.96	0.272	0.230	0.139
11	6.1	8	1.27	0.418	0.375	0.236
12	6.0	7	1.06	0.435	0.316	0.171
13	6.0	6	1.11	0.331	0.301	0.189
14	7.1	4	1.77	0.76	0.443	0.190
15	7.1	5	1.48	0.63	0.342	0.133
16	7.1	8	1.66	0.76	0.530	0.277
17*	6.9	5	2.04	0.85	0.465	0.180
18*	7.1	3	2.77	1.40	0.814	0.347
19	7.9	10	2.31	1.62	0.88	0.34
20	8.1	8	2.58	1.66	0.93	0.38
21	8.2	6	2.15	1.83	0.79	0.18
22	8.0	5	1.95	2.16	0.95	0.23
23*	8.1	4	2.71	2.57	1.27	0.41
24	9.0	10	3.27	2.78	1.36	0.43
25*	9.0	5	2.00	2.79	1.38	0.45
26	9.0	11	3.65	3.16	1.61	0.56
27	9.0	6	3.60	3.16	1.64	0.59
28	10.0	5	5.45	4.36	2.52	1.07
29	10.0	5	5.52	4.75	2.47	0.89
30	10.0	5	3.18	4.17	2.14	0.75
31	10.0	3	3.30	3.86	1.84	0.55
32*	9.9	5	3.90	5.46	2.32	0.50

TABLE III—*Concluded.*

Sample No.	Age.	No. of embryos.	Volume.	Uric acid.	Total nitrogen.	Residual nitrogen.
	<i>days</i>		<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
33	10.9	8	5.90	6.79	3.34	1.08
34	11.2	7	6.91	7.32	3.54	1.10
35	11.0	3	4.16	7.44	3.10	0.62
36	11.9	7	6.41	11.0	5.53	1.86
37*	12.0	6		10.8		
38	12.1	6	6.96	10.5	4.66	1.16
39*	12.0	3	5.64	9.9	4.08	0.79
40	12.0	4	3.99	10.1	3.76	0.41
41	12.9	8	5.19	15.1	6.44	1.41
42	12.9	5	6.16	14.1	6.95	2.25
43	13.1	6	6.68	14.1	7.56	2.86
44	13.1	5	8.32	17.1	8.94	3.26
45*	12.9	3	6.50	15.6	6.74	1.53
46*	13.9	7		17.2		
47*	13.9	6		21.6		
48*	14.1	3		18.4		

\* Omitted from the average (Table IV) for one or more of the following reasons: (1) data incomplete, (2) embryos not weighed, (3) embryos weighed more than 10 per cent above or below the average.

*Total Amount of Uric Acid in the Allantoic Fluid.*

As an index of protein consumption at any stage of embryonic life, it is naturally not the concentration but the total quantity of nitrogenous excretory products that must be considered. The method by which this information has been acquired we have given on a previous page. The individual results we now present in Table III, and the averages for each day in Table IV.

The allantoic fluid shows at first a fairly regular rise in volume, reaching by the end of the 11th day an average of 6 cc. From that time on there is apparently no change. These 6 cc. of fluid represent about 15 per cent of all the water in the egg; that much has been required to assist in the excretion of 7 mg. of uric acid. At the end of 19 days of incubation we have found as much as 100 mg. of uric acid in the allantoic cavity, and at that period very

little free fluid is in evidence. Water must be reabsorbed in some way, probably through the allantoic wall itself, and the process must begin at least as early as the 11th day, for after that the uric acid content of the allantoic fluid continues to increase without further rise in volume. The purpose of water reabsorption is self-evident; without it there appears to be no doubt that all the water that the egg contains, aside from that lost by evaporation,<sup>2</sup> would be needed to remove the uric acid. How closely the process as a whole is bound up with the properties of uric acid is so obvious that comment is almost superfluous. A substance as soluble and diffusible as urea, for example, could not possibly replace it as an

TABLE IV.  
*Average Composition of Allantoic Fluid.*

Age.	No of samples	No of embryos.	Volume.	Uric acid.	Total nitrogen.	Residual nitrogen.	Uric acid N. Total N.
<i>days</i>			<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
4.5	1	20	0.11	0.007	0.034	0.032	7
4.8	2	38	0.26	0.030	0.068	0.058	15
5	3	39	0.36	0.055	0.10	0.08	18
6	5	34	1.03	0.33	0.29	0.18	38
7	3	17	1.63	0.72	0.45	0.21	53
8	4	29	2.29	1.77	0.89	0.30	66
9	3	27	3.50	2.87	1.52	0.56	63
10	4	18	4.48	4.33	2.29	0.85	63
11	3	18	6.00	7.10	3.38	1.01	70
12	3	17	6.03	10.61	4.81	1.27	74
13	4	24	6.42	15.06	7.35	2.33	68

end-product when the organism and its excretions are confined to a closed system the wall of which is permeable only to matter in the gaseous state.

In connection with the quantitative aspects of nitrogen excretion in chick embryos, the question arises whether the main emphasis should be laid on the total nitrogen—as is usual in metabolism studies—or specifically on uric acid. We have chosen the latter for the following reasons. Reference to the figures for residual nitrogen (*i.e.* nitrogen not in the form of uric acid) in Table II will show that it has the characteristics of an incidental constituent of the allantoic fluid, rather than those of a true excretory product,

until nearly the end of the 2nd week of incubation. Before the 13th day the concentration of residual nitrogen is generally no higher than at the very start, while the concentration of uric acid has meanwhile increased 40 or 50 times. Moreover, a considerable proportion of the nitrogen not included under uric acid is present as amino acids, which cannot be regarded as waste products. The conclusion then appears to be inevitable that the residual nitrogen, until late in the 2nd week, represents in the main material that has reached the allantoic cavity by filtration, either through the kidney or through the allantoic wall.

*Rate of Protein Combustion in the Embryo.*

To get a rough idea of the extent to which protein participates in energy production we have proceeded in the following manner. First, the uric acid output for each day from the 5th to the 13th, inclusive, has been calculated by subtracting from the average total uric acid content of the allantoic fluid the corresponding figure for the day preceding. These results (Table V, Column 3) are readily converted into terms of protein.<sup>20</sup> From the amount of protein burned each day (Column 4) we have finally computed the yield of carbon dioxide derived from protein combustion (Column 5), taking 673 cc. of carbon dioxide (p. 537) as the amount obtained per gm. of protein burned. With the aid of Murray's<sup>2</sup> data for the carbon dioxide output of chick embryos (Column 6), it is now possible to estimate what fraction of the total carbon dioxide production has had its origin in protein (Column 7). The average for the entire period comes to 3 per cent. In other words, about 3 per cent of the energy is supplied by protein combustion.<sup>21</sup> The loss in total solids from the egg when the embryonic weight has reached 8.9 gm.—as in our specimens of 13 days—is about 500 mg.<sup>22</sup> At this stage an average of 15 mg. of uric acid has appeared in the allantois (Table IV), indicating the combustion of  $\frac{15 \times 6.25}{3} = 32$  mg. of protein. Therefore, in round numbers, 6 per cent of the material oxidized is protein.

<sup>20</sup> Uric acid  $\div$  3 = uric acid nitrogen; uric acid nitrogen  $\times$  6.25 = protein.

<sup>21</sup> Protein and fat yield about the same number of calories per liter of carbon dioxide when uric acid is the end-product of protein metabolism.

<sup>22</sup> Murray, H. A., Jr., *J. Gen. Physiol.*, 1925-26, ix, 405.

## 550 Nitrogen Metabolism in Chick Embryo

These calculations are of course beset with a number of uncertainties. For example, if we had used Bohr and Hasselbalch's data for total carbon dioxide production<sup>23</sup> in place of Murray's, the average ratio of protein CO<sub>2</sub> to total CO<sub>2</sub> would have come out a little higher. Also there is some doubt that the amount of carbon dioxide found experimentally exactly represents the amount that is produced.<sup>2</sup>

TABLE V.  
*Daily Protein Consumption by Chick Embryos.*

Day of incubation.	Average weight of embryos.	Daily uric acid output.	Protein equivalent of uric acid.*	CO <sub>2</sub> from protein.†	Total CO <sub>2</sub> production.‡	$\frac{\text{Protein CO}_2}{\text{Total CO}_2}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
	gm.	mg	mg.	cc.	cc.	per cent
5th	0.32	0.06±0.01	0.13	0.09		
6th	0.65	0.27±0.03	0.58	0.39	16	2.4
7th	1.02	0.39±0.04	0.81	0.55	21	2.6
8th	1.57	1.05±0.07	2.19	1.47	37	4.0
9th	2.32	1.10±0.09	2.29	1.54	56	2.8
10th	3.46	1.46±0.12	3.04	2.05	90	2.3
11th	4.91	2.77±0.15	5.77	3.88	120	3.2
12th	6.82	3.51±0.18	7.31	4.92	165	3.0
13th	8.86	4.45±0.51	9.27	6.24	180	3.5
Total for the entire 13 days .....		15.1	31.4	21.1	695	3.0

\* Uric acid nitrogen  $\times 6.25$ .

† Protein (mg.)  $\times 0.673$  (see p. 537).

‡ Data from Murray<sup>2</sup> by graphic interpolation, on the supposition that the total quantity of carbon dioxide produced since the beginning of the incubation period is a function of the embryonic weight.

Our estimate that 6 per cent of the substance burned during the first 2 weeks of incubation is protein is hence at best a rough approximation, but the order of magnitude must be correct. In one of his well known experiments, Siven<sup>24</sup> was able to establish nitrogen equilibrium in an adult man when only 6 per cent of the organic food material was protein. The two conditions are in a

<sup>23</sup> Bohr, C., and Hasselbalch, K. A., *Skand. Arch. Physiol.*, 1900, x, 149.

<sup>24</sup> Siven, V. O., *Skand. Arch. Physiol.*, 1900, x, 91.

sense analogous. While the daily protein "intake" in the case of the chick embryo is by no means low in relation to the body weight,—it is in the neighborhood of 20 gm. per kilo,<sup>22</sup>—yet the demand for protein for the building of new tissue is very great. It seems to us that protein combustion, far from being a virtually insignificant affair in chick embryo metabolism, is about as much concerned as in any organism where the supply of protein not required for repair or growth is reduced to its very lowest terms.

From the standpoint of the efficiency with which it utilizes protein for growth, on the other hand, the chick embryo does occupy an exceptional position. At the period when about 32 mg. of protein have been burned (*vide supra*) some 760 mg. of protein (according to the recent work of Murray<sup>22</sup>) have been transferred to the tissues of the embryo. Hence this transfer must have been accomplished with a waste of only 4 per cent; *i.e.*, no less than 96 per cent of the nitrogen taken from the food supply has been utilized for growth. As pointed out in the introductory discussion (p. 536), nothing approaching this efficiency has been attained during postembryonic life, at any rate in the mammalian organism.

#### *Irregularities in Uric Acid Output.*

Because of possible correlations with metabolic activity in general, as well as with events in the anatomical development of the excretory apparatus, attention should here be called to an unmistakable lack of regularity in the daily uric acid output. It is clear, from the figures given in Column 3 of Table V, that the gain in uric acid by the allantoic fluid abruptly rises on the 8th day, and again on the 11th, while at other times the rise is much more uniform. There is, to judge from Murray's observations (Column 6, Table V), a rather sharp increase in carbon dioxide production corresponding with the first of these irregularities. The second coincides, in point of time, with the beginning of what Needham<sup>1</sup> has described as a general "burst of chemical activity." At this stage also there appears to be an acceleration in the gain in dry weight by the embryo, attributable to more rapid synthesis of protein.<sup>22</sup> Increase in the rate of uric acid excretion associated with growth in the total traffic in protein material is exactly what one might expect. Further elaboration on these points will be



deferred to a forthcoming paper in which the anatomical aspects of the subject will also be included.<sup>9</sup>

Uric acid retention—such as might occur if the development of the excretory mechanism failed to keep pace with the demands upon it—will not account for the irregular course of the excretion of this product. We have determined the uric acid content of embryos of all ages between 5 and 11 days. While the individual figures for various reasons are hardly accurate enough to justify reporting them in full, yet we have no doubt that they are approximately right. They all lie between the limits of 1 and 2 mg. of uric acid per 100 gm. of tissue.

To get a satisfactory set of data on protein consumption during the final week of incubation would be a far more difficult matter, so with occasional exceptions we have limited our work to embryos of 13 days or less.

The few determinations of uric acid which we have made on the contents of the allantoic cavity in older embryos indicate that the variability is quite pronounced. Large numbers of analyses would naturally serve to circumvent this difficulty, but if that were done an even more serious obstacle would be met in attempting to interpret the results. Beginning about the 15th day, the phosphatides of the egg yolk begin to decompose. More strictly speaking, it is at that phase of development that the ether-soluble phosphorus of the entire egg contents begins to show a definite fall, and the inorganic phosphorus to rise—a process obviously connected with the calcification of the skeleton.<sup>25</sup> Hydrolysis of phosphatides necessarily sets free choline and amino ethyl alcohol, and possibly still other nitrogenous bases, the further fate of which is quite unknown. In the later days of incubation, then, to decide how much of the nitrogen entering the allantoic cavity has come from protein and how much from decomposing lipid material is at the present time impossible.

#### *Nitrogenous Products Other than Uric Acid.*

*Creatine.*—Scattered observations on the creatine content of the allantoic fluid have been made, primarily to determine whether this substance is concentrated by the embryonic kidney. At least before the 12th day this is evidently not the case, for the creatine concentration is uniformly in the vicinity of 5 mg. (equivalent to 2 mg. of nitrogen) per 100 cc. of fluid (Table VI), or about as much as blood would ordinarily contain. The fact that there is

<sup>25</sup> Plimmer, R. H. A., and Scott, F. H., *J. Physiol.*, 1909, xxxviii, 247; *J. Chem. Soc.*, 1908, xciii, 1700.

no less in the very early stages is of interest in connection with Mellanby's<sup>26</sup> failure to find a measurable amount of creatine in the embryo itself before the 14th day. Of course we cannot say with any degree of assurance that the chromogenic substance which we find actually is creatine; in fact this point is still to some extent in doubt with reference to the blood.<sup>27</sup> However, the chance that at least a portion of the color may be caused by creatine has led us to apply the color test to a number of tissue extracts obtained from embryos during the 2nd week of incubation. For the most part, these tests have been only qualitative or semiquantitative.

TABLE VI.  
*Creatine and Amino Acids in Allantoic Fluid.*

Age.	No. of embryos.	Concentration (per 100 cc.).				Total quantity per embryo.			
		Total N.	Uric acid N.	Creatine N.	Amino N.	Total N.	Uric acid N.	Creatine N.	Amino N.
<i>days</i>		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
4 8	20	26.7	3.8	0.4		0.082	0.011	0.001	
5.0	15	30.3	4.7	2.1	13.2	0.107	0.017	0.007	0.047
5.1	14	25.5	5.5	1.8	12.4	0.120	0.025	0.008	0.058
8.2	6	36.6	23.0	1.8					
8.2	4	46.9	31.7	1.5		1.27	0.86	0.039	
9.9	5	59.4	46.7		7.2	2.32	1.82		0.28
11.1	5		57.1	2.6					
12.0*	2	64.9	42.7	5.3					
12.9	3	102.6	79.7		7.3	6.74	5.21		0.47

\* Urea +  $\text{NH}_3\text{-N}$ , 4.7 mg. per 100 cc.

They were made on protein-free filtrates prepared for another purpose and not suitable for quantitative creatine determinations. With such material the picrate color test has been invariably positive, and indicates that creatine or a substance behaving in a similar way is present throughout the 2nd week in about the concentration found by Mellanby in later stages. Mellanby's negative results we are inclined to attribute to the fact that the color reaction, as used by him, is not delicate enough when the embryos are very small.

<sup>26</sup> Mellanby, E., *J. Physiol.*, 1907-08, xxxvi, 447.

<sup>27</sup> Behre, J. A., and Benedict, S. R., *J. Biol. Chem.*, 1922, lii, 11.

To the evidence provided by these rough tests we can add one quantitative observation. Two 8 day embryos were ground up with water and deproteinized by heat coagulation. A creatine determination on the filtrate, made by the autoclave method, showed 19 mg. per 100 gm. of tissue, or about two-thirds as much as Mellanby had found at 14 days. These few results, we think, suffice to dispose of Mellanby's hypothesis that the appearance of creatine in the tissues of the chick embryo is correlated with the development of the liver. Objections to Mellanby's interpretation have in fact been raised on other grounds by Needham.<sup>1</sup>

*Amino Nitrogen.*—The amino acids of the allantoic fluid are likewise present in about the concentration found in plasma (Table VI). Otherwise the chief point of interest about this fraction is that it accounts for approximately half the residual nitrogen at the 5 day stage, as well as in the one 10 day sample which was analyzed for amino nitrogen.

#### *Amniotic Fluid.*

*Total Nitrogen.*—In early embryos the total nitrogen content of the amniotic fluid is very low (Table VII). Beginning on the 12th day a sharp rise occurs, and by the 13th the fluid contains about 2.5 per cent of nitrogen. These results serve merely to give a quantitative aspect to the fact, long known to embryologists, that in the last half of the 2nd week a communication is established between the albumin sac and the amnion at the site of the seroamniotic junction.

*Uric Acid.*—Earlier in this paper we have mentioned, and incidentally disproved, the hypothesis of Aggazzotti\* that the allantoic fluid is not a true excretion. We can find no adequate support for still another opinion which the same author has expressed, that the non-volatile waste products of the embryo's metabolism are conducted to the amniotic cavity. Here again Aggazzotti has relied on pH determinations, unsupported by more substantial facts. Finding that the amniotic fluid becomes acid after the 11th day, and believing that the urine must be acid, he jumps to the conclusion that renal excretion does not begin until the 12th day, and that the excretory products then pass the allantois by and are stored in the amnion instead. The fact that no outlet from the cloaca into the amniotic cavity has appeared

until after the 17th day (Gasser) does not disturb him; he simply assumes the presence of a "functional communication."

We have not examined the amniotic fluid later than the 16th day, but up until that time it contains only a trace of uric acid (Table VII)—less than 0.1 per cent of the maximum concentration in the allantoic fluid, in spite of the thinness of the membrane separating the two cavities. During the first 16 days at least the allantois is the only "urinary bladder."

With one exception the uric acid figures for the amniotic fluid were obtained by precipitation with silver lactate, followed by extraction with an acid solution of sodium chloride and colorimetric

TABLE VII.  
*Total Nitrogen and Uric Acid in Amniotic Fluid.*

Age.	Amniotic fluid.		Average concentration of uric acid in allantoic fluid.
	Total N.	Uric acid.	
<i>days</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
6		0.31	32
7	2.9		44
9		0.30	82
10		0.38	97
11	9.5		
	5.6		
		0.34	118
12	156		176
13	2450		235
16	1500	0.52*	

\* Analysis of tungstic acid filtrate without silver precipitation.

analysis of the extract. The exception is the result recorded for the 16th day. In this instance protein had to be removed (with tungstic acid), and the protein-free filtrate was so dilute that silver precipitation was impossible. This one result is unquestionably high, for the direct colorimetric method has been tried on numerous samples obtained from younger embryos, and we know that one-half to two-thirds of the total chromogenic substance is not uric acid.

## SUMMARY.

1. Uric acid accumulates in the allantoic cavity of the chick embryo beginning at some time during the 5th day of incubation; *i.e.*, not long after the embryonic kidney has entered into communication with the allantois. The rate of excretion of uric acid is in general proportional to the body weight, but rises disproportionately on the 8th and the 11th days. By the end of the 13th day the allantoic fluid contains on the average 15 mg. of uric acid, more or less completely dissolved in about 6 cc. of water. By the end of the 19th day the total quantity excreted may reach 100 mg., now largely in the form of solid urate.

2. These facts suffice to prove (1) that the mesonephros is an active excretory organ, and (2) that protein is burned by the developing chick embryo to a significant degree, representing about 6 per cent of the organic matter oxidized during the first 2 weeks of incubation.

3. During the first 13 days of embryonic life about 96 per cent of the protein absorbed has been retained in the tissues of the embryo (including membranes).

4. Urate is unable to pass through the allantoic wall. This property and the low solubility of the salts of uric acid make the latter a substance peculiarly adapted to serve as the end-product of protein metabolism during embryonic life in animals which lay eggs surrounded by a shell.

5. The allantoic fluid likewise contains amino acids and creatine. The concentration of amino acids however does not rise as development proceeds, and the concentration of creatine (as well as that of undetermined nitrogen) remains approximately constant until about the 12th day. Consequently until that time uric acid is the only substance which can definitely be regarded as a true nitrogenous waste product.

6. Contrary to Mellanby's results, creatine is present in the tissues of the embryo at least as early as the end of the 8th day.

7. The amniotic cavity contains very little nitrogenous material until it is invaded by albumin; and at no time during the first 16 days of incubation more than a trace of uric acid. The nitrogenous excretions of the embryo are therefore confined to the allantois.

## THE FATE OF SUGAR IN THE ANIMAL BODY.

### II. THE RELATION BETWEEN SUGAR OXIDATION AND GLYCOGEN FORMATION IN NORMAL AND INSULINIZED RATS DURING THE ABSORPTION OF GLUCOSE.\*

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#### I. INTRODUCTION.

An outline of a quantitative analysis of the fate of ingested carbohydrate has been presented in the first paper (1) of this series. Such an analysis in the whole animal under physiological conditions seems of considerable interest, since one would learn what interrelations exist between sugar oxidation, glycogen formation, and fat formation. Before this problem could be attacked,

\* Presented in part before the American Society of Biological Chemists in Cleveland, Ohio, December, 1925.

## 558 Sugar Oxidation and Glycogen Formation

a suitable method of determining the absorption of the sugar had to be worked out. This method has been described in detail in the first paper. The next step was to adjust the experimental conditions in such a way that as much as possible of the absorbed sugar could be accounted for. Assuming that other metabolic processes besides oxidation, glycogen formation, and fat formation do not play a rôle in the disposal of sugar in the tissues, the amount of sugar recovered should depend on the accuracy of the methods used. The present experiments are an attempt to account for the glucose that has been absorbed during 4 hours by the normal and the insulinized rat. They deal only with the relation of sugar oxidation to glycogen formation, since fat formation did not occur. Experiments extending over longer periods, in which fat formation from glucose takes place, will be reported later.

### II. METHODS.

#### *1. General Plan of Experiments.*

The problem attacked by the experiments was to determine quantitatively on the same animal the nitrogen excretion in the urine, the sugar absorption, the sugar oxidation, and the glycogen formation. The general procedure was as follows:

Male rats of 120 to 150 gm. of body weight were used. They were reared in the laboratory on a ration that has been described previously (1). The animals were fasted for 48 hours in order to reduce the glycogen content of the tissues. The urine of the last 24 hours was collected quantitatively for nitrogen determinations. After 48 hours the animals were placed in the metabolism apparatus for a preliminary period of 3 hours. They were then fed a known amount of glucose solution by stomach tube or fed with glucose and injected with insulin (15 units per 100 gm. of body weight) and immediately replaced in the metabolism apparatus. After 4 hours the animals were stunned. The whole intestinal tract was quickly removed in order to determine the amount of sugar remaining in the intestine. The carcass was frozen with compressed  $\text{CO}_2$ , minced, and introduced into boiling KOH for glycogen determinations. The preformed glycogen was determined on a group of control rats, which were treated in exactly the same way with the exception of the sugar feeding. From the data thus obtained it can be calculated what percentage

of the absorbed sugar is accounted for by oxidation and glycogen formation. Further details about the experimental procedure will be found in the following sections.

The first set of experiments of the type just described was made in the fall and winter of 1925. It consisted of ten rats fed with glucose and of nine rats fed with glucose and injected with insulin. In addition, the glycogen content of eight control rats was determined. A second set of experiments was performed in the early spring of 1926 after the construction of a new metabolism apparatus. Here four experiments with glucose and two experiments with glucose plus insulin were made and the glycogen content of six control rats was determined. Since the results of both sets of experiments were the same, they can be recorded together. (See Tables II and III.)

## *2. Chemical Methods.*

Blood sugar was determined by the Hagedorn and Jensen method (2). All other sugar determinations were made by the Bertrand procedure. The permanganate solution used for the titrations was frequently standardized against C.P. glucose.

For glycogen, Pflueger's (3) prescriptions were followed. The tissues were hydrolyzed with 60 per cent KOH for 3 hours in the boiling water bath. The fluid was decanted from the bones that remained at the bottom of the flask. The bones were washed repeatedly with boiling water and the washings added to the KOH solution until the latter was diluted one-half. After precipitation with the double volume of 95 per cent alcohol, the samples were allowed to stand until the next day, when filtration was started. The bulk of the glycogen was collected on a small filter and was washed 3 times with 70 per cent, then with 90 per cent and absolute alcohol, then with ether, and finally with absolute alcohol. The glycogen was dissolved in water, HCl was added to make a 2.2 per cent solution, and the sample was heated for 3 hours at 100°C. in the water bath. Sugar was then determined in an aliquot part of the neutralized and filtered solution. Nerking (4) found in careful experiments that a loss of 3 per cent occurs when glycogen is hydrolyzed by HCl under the conditions just described. A correction of 3 per cent was, therefore, applied in all cases, the glycogen values in the tables being expressed in terms of glucose.



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Reference is made to the first paper as regards the method for the quantitative determination of sugar absorption. The amount of sugar fed minus the amount of sugar recovered from the whole intestinal tract represents the amount of sugar absorbed. 2.5 cc. of a 60 to 80 per cent glucose solution, warmed up to 39°C., was given by stomach tube. Enough sugar was introduced in this way to let the absorption proceed for 5 to 6 hours. Immediately before and after the feeding of each rat, the same amount of sugar solution that was fed, was delivered into volumetric flasks. These two deliveries generally checked within 1 to 2 per cent.

For the collection of urine the bladder was emptied at the beginning and the end of the 24 hour period. The rats were kept in small wire screen cages, which were placed on plates. As a preservative 1 per cent  $\text{H}_2\text{SO}_4$  was added. The nitrogen was determined by the Kjeldahl procedure.

### *3. Measurement of the Respiratory Exchange.*

For the first set of experiments Haldane's method (5) was used for the determination of the respiratory exchange. The metabolism apparatus was set up in an electrically regulated warm room, where the temperature was kept at 27°C. with a variation of  $\pm 0.8^\circ$ . The rats were placed in the warm room 24 hours before beginning the experiments. The animal chamber consisted of a large fruit jar of 1700 cc. capacity and was kept air-tight by a rubber stopper and two clamps. In- and outlet tubes had a diameter of 8 mm., the one extending to the bottom of the chamber the other having its opening at the top of the chamber. The animal chamber was also fitted with a thermometer, graduated in 0.1°C. In all experiments the temperature of the chamber was kept between 27 and 28°C. Since the volume of the chamber was known, corrections for changes in temperature and barometric pressure could be applied. For the water and  $\text{CO}_2$  absorption wash bottles of 300 cc. capacity with ground glass stoppers were used. The water absorbers were half filled with concentrated  $\text{H}_2\text{SO}_4$ . The  $\text{CO}_2$  absorbers contained Wilson's soda lime. Compressed air, previously freed from  $\text{CO}_2$  and water, was passed through the system at a rate of 120 to 200 liters per hour. The animal chamber and the containers were weighed on a Troemner scale of 2 kilos capacity and a sensitivity of  $\pm 1$  mg. The scale was in the same room as the metabolism apparatus.

The second set of experiments was performed with a closed circuit type of metabolism apparatus, constructed after the principle of Fridericia (6). This apparatus combines the Haldane method with a direct oxygen determination. The great advantage is that each metabolism experiment is at the same time a control experiment for the proper functioning of the apparatus, since the oxygen is determined by two different methods. A leak or an incomplete absorption of  $\text{CO}_2$  or water is immediately detected. Since this apparatus will be used for later work, it is described in detail below. A small Cenco blower, which is immersed in oil, circulates the air at a rate of 120 to 200 liters per hour. The blower is connected by gears with an electric motor, the speed of which is controlled by a rheostat. The air passes through the animal chamber and through the water and  $\text{CO}_2$  absorbers and returns through two wash bottles, fitted with thermometers graduated in  $0.1^\circ\text{C}$ . The second bottle serves as manometer and registers the pressure in the apparatus. With this closed system there is connected a spirometer from which the oxygen is drawn automatically, as soon as the pressure in the system falls, due to the intake of oxygen by the animal. The spirometer consists of a bell of narrow and thin aluminum tubing and of a larger outside mantle, filled with water. An outlet tube extends slightly above the surface of the water. The water seal is kept at a constant level by an overflow device, attached to a suction pump. The spirometer bell is suspended on a silk thread, which passes over an aluminum wheel with ball bearings. A copper cylinder filled with water serves as a counterpoise. The copper cylinder communicates by thin rubber tubing with a long burette and equilibrates the spirometer bell in all positions by the principle of communicating vessels. The outlet tube of the spirometer is connected with a two arm manometer, which must always register atmospheric pressure. A wash bottle filled with  $\text{H}_2\text{SO}_4$  dries the oxygen before it enters the apparatus. The spirometer bell is fitted with a thermometer graduated in  $0.1^\circ\text{C}$ . and a pointer which slides along a mm. scale. 1 mm. of the scale corresponds to approximately 1 cc. The capacity is about 1 liter. The spirometer readings have, of course, to be reduced to  $0^\circ\text{C}$ ., 760 mm., and dryness. Since the volume of the rest of the apparatus is known, corrections for changes in temperature and barometric pressure

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can easily be applied. In order to obtain satisfactory results, the inertia of the spirometer must be as small as possible.

Up to the present forty metabolism experiments have been made with the closed circuit apparatus. The agreement between the direct and indirect oxygen determination for 3 to 4 hour periods was, as an average, 1.4 per cent with an average deviation for a single experiment of  $\pm 0.7$  per cent. The difference in the respiratory quotient, when calculated from the direct and indirect oxygen value, was, as an average, 0.010 with an average deviation for a single experiment of  $\pm 0.006$ . The respiratory quotients obtained with our apparatus are, therefore, accurate within at least  $\pm 0.008$ . These results show incidentally that the Haldane method is very reliable and accurate when properly executed. It seemed advantageous to keep the heat production of the rats as low as possible. In order to achieve this end, the surrounding temperature had to be sufficiently high and the animals had to remain perfectly quiet. It has been mentioned previously that the animals were kept between 27 and 28°C. for 24 hours before the start as well as during the metabolism experiment. At this temperature the  $O_2$  consumption of rats is at its lowest level. Furthermore, rats made tame and gentle by frequent handling are ideal animals for respiratory metabolism work, since they sleep most of the time, provided the air current passing through the animal chamber is not too strong. Occasionally they clean themselves between periods of sleeping, but this lasts only for 2 to 4 minutes. Otherwise they remain very quiet when they are awake. Especially after the sugar feeding the animals slept for practically the whole period of 4 hours.

### III. EXPERIMENTAL.

#### *1. The Glycogen Content of Rats Fasted for 48 Hours.*

It is evident that the absorbed glucose could not be satisfactorily accounted for if the preformed glycogen of the rats would vary within wide limits. The latter has to be subtracted in each case in order to obtain the amount of glycogen actually formed during the period of sugar absorption. The control rats on which the preformed glycogen was determined, were evenly distributed over the experimental period. Since the intestine could not be included in the glycogen determinations of the metabolism rats, the control rats were also worked up without intestine.

Table I shows that the glycogen content of the control rats is very constant. The average deviation from the mean of 0.114 per cent is only  $\pm 11$  mg. of glycogen. Using the maximum and minimum figure for the calculation of the results of Experiment 1 in Table II, the recovery of the absorbed sugar would be 91.6 and 94.5 per cent respectively. The error introduced by variations in the preformed glycogen is, therefore, small.

TABLE I.  
*Glycogen Content of Male Rats, Fasted Previously for 48 Hours.*

Body weight.	Total glycogen.	Glycogen in per cent of body weight.	Remarks.
<i>gm.</i>	<i>gm.</i>		
137.9	0.163	0.118	First series of experiments.
136.1	0.128	0.094	" " " "
124.0	0.156	0.126	" " " "
144.6	0.184	0.127	" " " "
147.5	0.138	0.093	" " " "
129.5	0.159	0.123	" " " "
133.3	0.144	0.108	" " " "
116.4	0.120	0.103	" " " "
122.9	0.115	0.094	Second series of experiments.
95.9	0.114	0.119	" " " "
110.1	0.139	0.126	" " " "
127.2	0.155	0.122	" " " "
114.8	0.147	0.128	" " " "
109.6	0.126	0.115	" " " "
Average.....		0.114	

In the following paper values for the glycogen content of the liver of control rats are given. Calculated per 100 gm. of body weight, the liver corresponds to 2.6 per cent of the body weight and contains as an average  $10.3 \pm 2.4$  mg. of glycogen. The liver is, therefore, only to a small extent responsible for the variations that occur in the glycogen content of the whole body. This is due to the fact that the liver of rats fasted for 48 hours contains only 9 per cent of the total glycogen stores of the body.

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### 2. The Nitrogen Excretion of Fasting Rats.

The relative amounts of fat and carbohydrates oxidized were calculated from the non-protein respiratory quotient, using 8.488 mg. of O<sub>2</sub> as the equivalent of 1 mg. of urine N. The nitrogen excreted during the 4 hours of sugar absorption could not be determined directly, but was calculated from the nitrogen values

TABLE II.

*Summary of the Recovery Experiments on Normal Animals.*

All values are per 100 gm. of body weight per 4 hours.

Glucose absorbed.	Glucose oxidized.	Glycogen formed.	Glucose recovered.		Glucose lost	Glycogen formed Glucose oxidized	Blood sugar.	O <sub>2</sub>	CO <sub>2</sub>	R.Q.	Urine N.
gm.	gm.	gm.	gm.	per cent	gm.		mg.	gm.	gm.		mg.
0.746	0.384	0.310	0.694	93.1	0.052	0.81	147	0.736	0.895	0.883	12.17
0.764	0.366	0.335	0.701	91.4	0.063	0.91	173	0.751	0.867	0.840	12.06
0.729	0.360	0.366	0.726	98.4	0.003	1.01	178	0.815	0.960	0.856	11.21
0.666	0.273	0.304	0.577	86.7	0.089	1.11	169	0.634	0.749	0.859	12.92
0.698	0.266	0.354	0.620	88.7	0.078	1.33	163	0.695	0.800	0.838	10.21
0.674	0.279	0.396	0.674	100.0	0.000	1.42	164	0.801	0.997	0.832	12.96
0.856	0.298	0.437	0.735	85.8	0.121	1.47	181	0.747	0.869	0.846	12.92
0.751	0.280	0.412	0.692	92.3	0.059	1.48	182	0.676*	0.792	0.849	13.08
0.757	0.236	0.373	0.609	80.4	0.148	1.58	185	0.689	0.784	0.827	10.71
0.701	0.239	0.381	0.620	88.5	0.081	1.59	171	0.771	0.866	0.817	12.30
0.785	0.241	0.399	0.640	81.5	0.145	1.66	175	0.707*	0.806	0.827	11.56
0.850	0.296	0.498	0.794	93.7	0.056	1.68	179	0.772*	0.897	0.845	12.30
0.684	0.204	0.367	0.571	83.6	0.113	1.80	180	0.776	0.857	0.802	13.00
0.836	0.210	0.495	0.705	84.4	0.131	2.30	198	0.764*	0.906	0.807	12.96

\* O<sub>2</sub> determined by direct and indirect method.

obtained for the preceding 24 hours. The rats were weighed at the beginning and the end of the 24 hour period. Based on the average weight during this period, the amount of nitrogen excreted per 100 gm. of body weight per hour was found. This figure, diminished by 3 per cent, was then used to calculate the nitrogen excretion during the sugar absorption period. This procedure seemed permissible, since it was found in several experiments that the nitrogen excretion of fasting rats diminishes parallel to the

decrease in body weight. The result is that the N excretion per 100 gm. of body weight per hour for consecutive periods of fasting is a fairly constant value. An example of this is given below. Average weight of rat between the 24th and 32nd hour of fasting, 151.9 gm. Total N excreted, 36.05 mg. N per 100 gm. of body weight per hour, 2.96 mg. Average weight of rat between 32nd and 48th hour of fasting, 147 gm. Total N excreted, 73.35 mg. N per 100 gm. of body weight per hour, 3.11 mg.

TABLE III.

*Summary of Recovery Experiments on Insulinized Animals.*

All values are per 100 gm. of body weight per 4 hours. 15 units of insulin per 100 gm. of body weight were injected simultaneously with the sugar feeding.

Glucose absorbed.	Glucose oxidized.	Glycogen formed.	Glucose recovered.		Glucose lost.	Glycogen formed Glucose oxidized	Blood sugar.	O <sub>2</sub>	CO <sub>2</sub>	R.Q.	Urine N.
gm.	gm.	gm.	gm.	per cent	gm.		mg.	gm.	gm.		mg.
0.693	0.403	0.273	0.676	97.5	0.017	0.68	55	0.671	0.841	0.911	13.08
0.823	0.401	0.284	0.685	83.3	0.138	0.71	75	0.631	0.803	0.925	13.54
0.729	0.382	0.279	0.661	90.6	0.068	0.73	68	0.748*	0.876	0.850	11.17
0.690	0.390	0.318	0.708	102.6	0.000	0.82	69	0.696	0.859	0.894	12.53
0.801	0.393	0.339	0.732	91.4	0.069	0.86	57	0.706	0.870	0.894	12.60
0.834	0.419	0.376	0.795	95.2	0.039	0.90	71	0.736*	0.914	0.901	12.92
0.846	0.428	0.386	0.814	96.1	0.032	0.90	52	0.811	0.987	0.883	11.17
0.812	0.392	0.357	0.749	92.2	0.063	0.91	83	0.740	0.913	0.886	11.21
0.764	0.330	0.307	0.637	83.4	0.127	0.93	129	0.795	0.931	0.851	13.08
0.664	0.249	0.317	0.566	85.1	0.098	1.27	107	0.764	0.866	0.886	12.11

\* O<sub>2</sub> determined by direct and indirect method.

The correction of 3 per cent had to be applied, since the ingestion of carbohydrates has a sparing action on the protein metabolism. It was found in previous experiments (1) that during 5 hours of glucose absorption or glucose absorption plus insulin, the nitrogen excretion per 100 gm. of body weight per hour diminished by 4.9 and 5.7 per cent respectively. In a new set of experiments, the nitrogen excretion was found to decrease by 3 per cent, when the absorption was allowed to proceed for only 4 hours.

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The following are the nitrogen values that were found on the rats used for the experiments in Tables II and III. N of the last 24 hours calculated per 100 gm. of body weight per hour: 3.14, 3.11, 2.89, 3.33, 2.63, 3.34, 3.33, 3.37, 2.76, 3.16, 2.98, 3.17, 3.35, 3.37, 3.12, 3.49, 2.88, 3.23, 3.25, 3.33, 2.87, 2.89, 3.37, 3.12. Average,  $3.18 \pm 0.20$  mg.

The larger variations are probably due to the fact that the bladder of such a small animal as the rat cannot always be completely emptied. However, the error thus introduced is small, since in fasting rats only 10 per cent of the total calories are derived from protein. Using again the maximum and minimum figure for the calculation of the results of Experiment 1 in Table II, the recovery of the absorbed sugar would be 92.9 and 93.4 per cent respectively.

### *3. The Respiratory Quotient of Fasting Rats.*

The following are the non-protein respiratory quotients of the experimental rats before sugar was fed: 0.719, 0.705, 0.718, 0.708, 0.715, 0.716, 0.714, 0.713, 0.709, 0.715, 0.717, 0.703, 0.717, 0.715, 0.714, 0.701, 0.708, 0.703, 0.717, 0.705, 0.706, 0.704, 0.706, 0.713. Average,  $0.711 \pm 0.005$ .

These quotients indicate that after a 48 hour fast 99 per cent of the non-protein calories are derived from fat or that a negligible amount of carbohydrates is being oxidized. In four instances rats with higher quotients were found, which were for this reason unsuitable for the experiments. Among the forty-nine fasting quotients, observed from October until May, three were below 0.700. From numerous additional experiments that have recently been made, these low quotients must be attributed to acidosis. Rats excreting acetone bodies cannot of course, be used for sugar recovery experiments. The oxidation of the acetone bodies that occurs after the sugar feeding makes it impossible to calculate the amount of carbohydrate oxidized from the respiratory quotient. Interestingly enough, fasting quotients below 0.700, associated with strong acidosis, are the rule during the summer months. These seasonal changes in the fat metabolism of the rat will be described in a later communication.

4. *The Relation between Sugar Oxidation and Glycogen Formation in Normal and Insulinized Rats.*

The averages of all experiments have been calculated and are given in Table IV. It is proposed to discuss this table first, before

TABLE IV.  
*Average of Experiments in Tables II and III.*

All values are per 100 gm. of body weight per 4 hours.

	Glucose alone (14 rats).	Glucose plus insulin (10 rats).
Glucose absorbed.....	0.750 gm.	0.766 gm.
"    oxidized.....	0.281 "	0.378 "
Glycogen formed.....	0.388 "	0.324 "
Glucose recovered.....	0.669 "	0.702 "
"    lost.....	(89.2 per cent). 0.081 gm.	(91.6 per cent). 0.064 gm.
Glycogen formed		
Glucose oxidized.....	1.38	0.87
Blood sugar.....	0.176 gm.	0.077 gm.
CO <sub>2</sub> .....	0.851 "	0.886 "
O <sub>2</sub> .....	0.738 "	0.730 "
R.Q.....	0.838	0.882
Urine N... ..	12.15 mg.	12.3 mg.
Non-protein CO <sub>2</sub> .....	0.737 gm.	0.742 gm.
"    O <sub>2</sub> .....	0.635 "	0.600 "
"    R.Q.....	0.844	0.899
Protein oxidized. ....	0.075 gm.	0.076 gm.
Fat oxidized.....	0.118 "	0.072 "
Calories from protein*.....	0.30	0.31
"    "    fat.†.....	1.11	0.68
"    "    glucose.‡.....	1.05	1.41
Total calories.....	2.46	2.40

\* Heat from protein (urine N  $\times$  24.98 calories).

† Heat value for "animal fat" 9.4 calories.

‡ Heat value for glucose 3.74 calories.

the individual experiments are analyzed. It will be noted that in the series with glucose alone, 89 per cent of the absorbed sugar was recovered. The 11 per cent that is not accounted for corresponds to a loss of 81 mg. of glucose. However, a determination of the glucose concentration in the tissues and of the intermediary car-



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bohydrates has not been included. Based on previous observations (7), we assume that the increase in the glucose content of the tissues, as the result of the increased blood sugar level, would amount to approximately 30 mg. The remaining loss of 51 mg. or of 6.8 per cent may be attributed partly to the formation of intermediary carbohydrates, partly to a postmortem disintegration of glycogen. In the experiments with glucose plus insulin the average amount of sugar recovered was 91 per cent. Since the insulin injection keeps the blood sugar at a low level and also tends to reduce the glucose content of certain tissues (7), the loss of 64 mg. of glucose may be entirely attributed to the formation of intermediary carbohydrates and to postmortem glycogenolysis.

Table IV also shows that the average amount of sugar absorbed was nearly the same in both series. This indicates that insulin has no influence on the rate of absorption of sugar from the intestine.

The essential feature of the experiments is that both the normal and the insulinized animals have the same amount of sugar available. As long as this experimental condition is not fulfilled, quantitative data on the effect of an insulin injection cannot be obtained. The next point is that in both groups of animals 90 per cent of the absorbed sugar is accounted for by glucose oxidation plus glycogen formation. It may be concluded from this that an excess of insulin does not produce qualitative changes in carbohydrate metabolism. The merely quantitative nature of the change is illustrated by the quotient  $\frac{\text{glycogen formed}}{\text{glucose oxidized}}$ . This

quotient indicates how many mols of glucose are polymerized into glycogen, when 1 mol of glucose is oxidized. In the insulin series the average quotient is 0.87 as compared with 1.38 for the normal animals. Expressed in other terms it means that the rats receiving glucose plus insulin oxidize 97 mg. more glucose and deposit 64 mg. less glycogen than the rats receiving glucose alone. The obvious conclusion is that an excess of insulin over that produced by the normal animal increases the sugar oxidation. From this it follows that the amount of sugar oxidized in the normal animal depends on the amount of hormone released by the pancreas. A further deduction is that the sugar oxidation is the primary process and the glycogen formation the secondary phenomenon. In drawing these conclusions it is realized that it is

not known through what intermediary stages the glucose passes before it is oxidized into  $\text{CO}_2$  and water.

It is of interest that the total calories produced by the normal and insulinized animals were nearly the same, as is shown in Table IV. The calories derived from protein were equal in both groups of animals. The increased sugar oxidation in the insulinized animals was counterbalanced by a decrease in fat oxidation in equicaloric proportion.

The individual experiments, which are shown in Figs. 1 and 2 in the form of a bar diagram may now be considered. All values

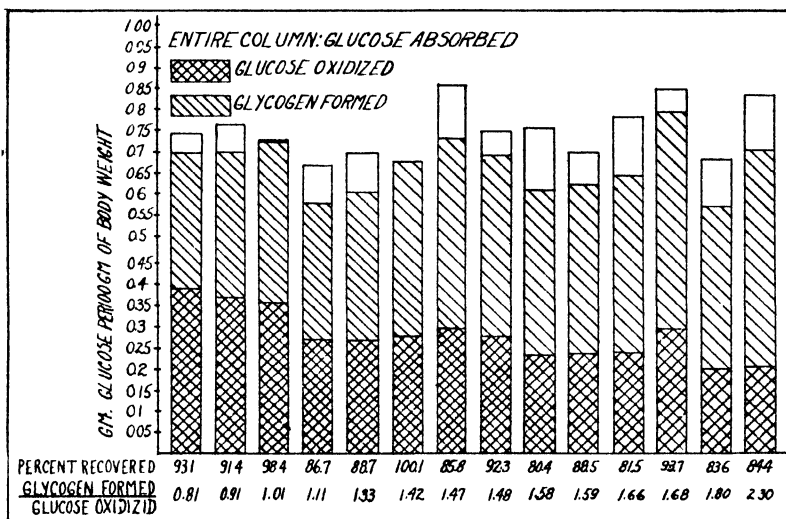


FIG. 1. Graphic representation of the data of Table II (normal animals).

are per 100 gm. of body weight. The entire length of the columns represents the amount of sugar absorbed during 4 hours. The cross-hatched and the obliquely shaded portions of the columns correspond to the amount of glucose oxidized and the amount converted into glycogen, respectively. The blank spaces represent the glucose that has not been accounted for. The figures below each column indicate the percentage of the absorbed sugar that has been accounted for and the quotients  $\frac{\text{glycogen formed}}{\text{glucose oxidized}}$ . Finally, the columns are so arranged that the quotients increase from left to right.

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In the normal animals the amount of glucose oxidized per 100 gm. of body weight per 4 hours shows a considerable individual variability. The values range from 0.204 to 0.384 gm., which corresponds to a difference of 47 per cent. This was not due to bodily movements or to changes in the outside temperature, since these two factors were carefully controlled. However, in the insulinized animals the sugar oxidation remains remarkably constant. The values in the first eight experiments vary only from 0.382 to 0.428 gm. or by 11 per cent. This constancy may be

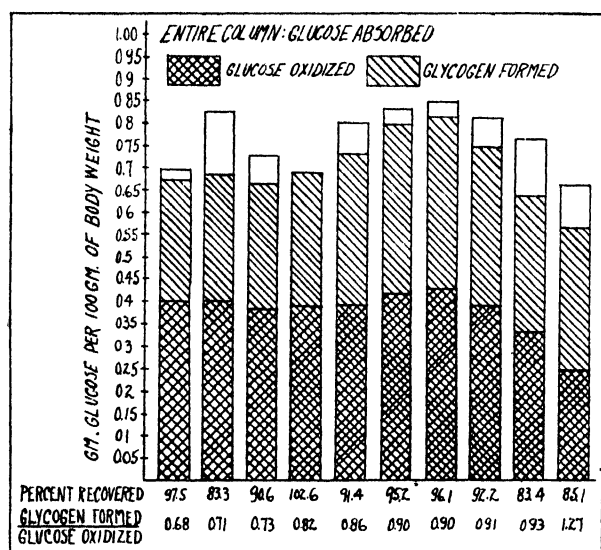


FIG. 2. Graphic representation of the data of Table III (insulinized animals).

explained in the following way. There is obviously a limit to the rate at which sugar can be oxidized in the body. Under the conditions of our insulin experiments this limit is reached, that is to say, the sugar oxidation proceeds at its maximum rate. This maximum rate in any given animal at a given sugar concentration in the tissues will be a function of the mass of active tissue, provided there is an excess of insulin present. Since we are dealing with a uniform animal material, the mass of active tissue will be proportional to the body weight. The constancy of the above results, in terms of unit of body weight, is, therefore, not surprising.

The maximum rate of sugar oxidation is of significance for the study of carbohydrate metabolism, since it represents a well defined experimental condition. We propose to speak of completely insulinized animals whenever this condition is attained. The opposite would be the completely diabetic animal, where the rate of sugar oxidation approaches zero. As a rule, the normal animal falls somewhere between these two extremes. It is, therefore, of interest that one of the normal animals (Experiment 1, Table II) reaches the maximum rate of sugar oxidation and that two of the insulinized animals (the last two experiments in Table III) fall below this rate. The former case shows that the pancreas of rats may be capable of releasing enough insulin to produce a maximum hormone effect. This explains the small difference in the glucose tolerance of normal and insulinized rats, as is discussed in the fourth paper of this series. In the latter case the animals were apparently incompletely insulinized, which is also shown by the fact that the blood sugar values were higher than in the other experiments.

#### *5. The Rôle of the Liver and the Muscles in the Disposal of Sugar.*

It has been shown in the preceding section that the increased sugar oxidation in the insulinized animals was balanced by a decreased glycogen deposition. It remained to be determined in which organs the increased sugar oxidation occurred and which organs were responsible for the decrease in glycogen formation. Previous work (8) pointed to the liver as the organ where less glycogen was deposited. A study of the glycogen formation in the liver under conditions analogous to the experiments here reported was, therefore, undertaken. The details of this investigation are recorded in the following paper. It was found that the difference in the glycogen content of normal and insulinized animals was entirely due to the liver. This is illustrated in Table V. Thus the difference in the total glycogen of the normal and insulinized animals amounts to 64 mg., while the difference in the liver glycogen amounts to 83 mg. This leaves a plus of 19 mg. for the rest of the body tissues, which may be due to an actual increase in the muscle glycogen of the insulinized animals over that of the normal animals.

The question where the increased sugar oxidation in the in-

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sulinized animal occurs, is more difficult to answer. It is not probable that the liver oxidizes the glucose instead of polymerizing it into glycogen, since this would necessitate an increase in the metabolism of the liver of about 150 per cent. It has been found previously (9) on sugar-fed rabbits by a comparison of the sugar concentration in arterial and venous blood that insulin increases the sugar intake of the muscles. Since the muscles are responsible for about 50 per cent of the total metabolism, it is very likely that the increased sugar oxidation occurs chiefly in them.

These results can hardly be interpreted in the sense that insulin inhibits glycogen formation in the liver, since the normal sugar-

TABLE V

*Amount of Glycogen Formed in Liver and in Rest of Body during 4 Hours of Glucose Absorption.*

Calculated from Table IV of this paper and from Table II of the following paper. All values are per 100 gm. of body weight. The preformed glycogen has been subtracted in each case.

	Liver in per cent of body weight.	Glycogen in liver.	Glycogen in other tissues.	Total glycogen.	Liver in per cent of total.	Other tissues in per cent of total.
		gm.	gm.	gm.		
Glucose alone .....	3.22	0.118	0.270	0.388	30.0	70.0
“ plus insulin.....	3.06	0.035	0.289	0.324	10.8	89.2
Difference.....		-0.083	+0.019	-0.064	-19.2	+19.2

fed animals, with the aid of their own insulin production, deposited considerable amounts of liver glycogen. This effect of an excess of hormone is probably of a secondary nature and is due to the increased disposal of sugar in the muscles. The muscles appropriate so much sugar that there is nothing left to be stored in the liver. (It is the same process that leads to a depletion of the glycogen stores of the liver in fasting insulinized animals.) There exists, therefore, a competition between liver and muscles for the absorbed glucose, and with an excess of insulin the muscles remain victorious.

## IV. DISCUSSION.

Macleod (10) advanced the hypothesis that part of the sugar that disappears in the normal animal following an insulin injection is converted into an unknown substance. He arrived at this conception, partly because less glycogen was deposited in the liver, partly because the oxidation did not seem to account for all the sugar that disappeared. His hypothesis was, however, not based on quantitative data. In our present experiments an unknown substance is not formed, since in both the normal and the insulinized animals 90 per cent of the sugar that disappears is accounted for. It has already been reported in the first paper that the rate of absorption of glucose from the intestine of the rat is twice as large as the intravenous tolerance rate of rabbits, dogs, and men. The amount of sugar metabolized in our experiments was, therefore, very large. Nevertheless, there remained the possibility that more sugar could be administered by the intravenous route than could be absorbed and that an unknown substance was formed under these conditions. In the fourth paper of this series a detailed study of the intravenous tolerance of normal and insulinized animals will be reported. What interests us here is the fact that the rate at which glucose can be infused intravenously into rats without causing glucosuria is not much greater than the rate at which glucose is absorbed from the intestine. Furthermore, insulin raised the intravenous tolerance only to a slight extent. For these reasons we have desisted from repeating our experiments during the intravenous infusion of sugar.

Bissinger and Lesser (11) recently reached a similar conclusion; namely, that Macleod's hypothesis cannot be proven experimentally. Lesser and coworkers made three series of experiments. In the first series (12) they injected glucose intraperitoneally into normal and insulinized mice. By analyzing the whole body of control mice and of the injected mice for free carbohydrates and for glycogen, they could calculate how much of the injected sugar had disappeared. In a second series (13) Lesser determined the gaseous metabolism of the mice in order to find out how much of the sugar that had disappeared was accounted for by oxidation. The recovery was, however, not satisfactory owing to the small number of mice involved in the first series. Bissinger and Lesser (11) repeated, therefore, the

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first series using 360 mice in all. Their results may be summarized as follows: 30 minutes after the injection of glucose plus insulin  $141 \pm 6$  mg. of sugar per 100 gm. of body weight had disappeared. Of these 128 mg. were accounted for by oxidation, corresponding to a recovery of 91 per cent. 40 minutes after the injection  $155 \pm 5$  mg. had disappeared, while 159 were accounted for by oxidation. Here the recovery amounted to 100 per cent.

Lesser believes that glucose oxidation and glycogen formation is a coupled reaction, which is accelerated by insulin. In analogy to the Meyerhof quotient of the muscle he divides the total amount of glucose that has disappeared by the glucose that has been oxidized. This quotient equals 1.27 in his experiments. Lesser calculated from the experiments of Burn and Dale (14) and of Ringer (15) quotients of 1.6 to 1.7. In the experiments presented in this paper the quotients for the normal animals vary from 1.9 to 4 with an average of 2.7. If the average quotient for the muscles alone is calculated, which may be done on the assumption that the R. Q. of the muscles is close to the R. Q. of the whole animal (Himwich and Castle (16)) and that the muscles are responsible for 50 per cent of the metabolism of the body, a value of 2.9 is obtained. The quotients for the insulinized animals are lower, due to the increased sugar oxidation. They range from 1.7 to 2.3 with an average of 2.0. Meyerhof found for the frog muscle under a variety of conditions quotients  $\frac{\text{lactic acid disappeared}}{\text{lactic acid oxidized}}$

of 3 to 6. Warburg, Posener, and Negelein (17) recently found quotients of equal magnitude for a number of "glycolyzing" mammalian tissues. In the recovery process of the muscle the energy set free by the oxidation of lactic acid is used for the re-conversion of lactic acid into glycogen. The higher the quotients the greater is the efficiency of the recovery process. It will be noted that the quotients  $\frac{\text{glucose disappeared}}{\text{glucose oxidized}}$  are lower than the Meyerhof quotients for the isolated tissues. One would expect the opposite, since the efficiency of synthetic processes should be higher in the intact animal than in tissues surviving *in vitro*. It is possible that the glycogen is split into glucose as soon as it is formed and that the glucose is again reconverted, and so on, which would account for the low quotients. On the other hand, it may

not be correct to compare glucose oxidation and polimerization with lactic acid oxidation and glycogen formation from lactic acid. There is at least no indication at present that glucose in the intact animal is split into lactic acid before it is either oxidized or converted into glycogen.

#### V. SUMMARY AND CONCLUSIONS.

1. The fate of ingested glucose has been followed quantitatively by determining on the same animal the amount of sugar absorbed, oxidized, and stored as glycogen.

2. Glucose oxidation and glycogen formation accounted for 90 per cent of the sugar absorbed during 4 hours in both normal and insulinized animals.

3. The quotient  $\frac{\text{glycogen formed}}{\text{glucose oxidized}}$  was 1.38 for the normal rats and 0.87 for the insulinized rats. Since both groups of animals absorbed the same amount of sugar, this indicates that the insulinized rats oxidized more glucose and deposited less glycogen than the normal rats. The difference in the liver glycogen of the normal and insulinized rats accounted for the difference in the total glycogen. Therefore, the insulinized animals deposited in the rest of the body tissues (chiefly in the muscles) the same amounts or even more glycogen than the normal animals.

4. It seems probable that the increased sugar oxidation in the insulinized animals occurs chiefly in the muscles.

5. It is concluded from these experiments that an excess of insulin leads to an increased sugar oxidation.

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## THE FATE OF SUGAR IN THE ANIMAL BODY.

### III. THE RATE OF GLYCOGEN FORMATION IN THE LIVER OF NORMAL AND INSULINIZED RATS DURING THE ABSORPTION OF GLUCOSE, FRUCTOSE, AND GALACTOSE.

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(Received for publication, August 26, 1926.)

The experiments of the preceding paper give a clear picture of the fate of ingested glucose. In the normal animal during 4 hours of sugar absorption for 100 parts of the absorbed sugar, 38 parts are oxidized, while 36 parts are deposited as glycogen in the muscles and 16 parts in the liver. In the insulinized animals for 100 parts of absorbed sugar, 50 parts are oxidized, 38 parts are deposited in the muscles, and only 4 parts in the liver. It seemed of interest to supplement these findings by a detailed study of the glycogen formation in the liver at shorter and longer absorption periods and to include other sugars. In this way curves for the rate of glycogen formation in the liver could be constructed and the percentage of the absorbed sugar that was retained as liver glycogen could be calculated.

#### EXPERIMENTAL.

The experiments were made in the fall of 1925 and in the winter of 1926. Male rats were used that weighed between 120 and 150 gm. The animals were fasted for 48 hours in order to reduce the glycogen content of the liver. They were then fed a known amount of sugar solution by stomach tube or fed with sugar and injected with insulin (15 units per 100 gm. of body weight). Enough sugar was introduced to let the absorption proceed for 6 hours. Groups of 4 to 6 rats were killed 1, 2, 3, 4, and 5 hours after the sugar feeding. The whole liver was removed as quickly as possible, frozen with compressed CO<sub>2</sub>, weighed in the frozen state, minced, and introduced into boiling 60 per cent

KOH. Glycogen was then determined by Pflueger's method. The amount of sugar absorbed was determined in the manner described in the first paper (1).

The glycogen values obtained during the absorption of sugar were corrected for the preformed glycogen. Table I shows that the preformed glycogen, determined on suitable control rats, was, as an average, 0.397 per cent. This figure was subtracted in each case. The variation in the preformed glycogen of  $\pm 0.090$  per cent introduces no serious error, since the amount of glycogen deposited in the course of the sugar absorption is large in comparison.

TABLE I.

*Glycogen Content of the Liver of Male Rats, Fasted Previously for 48 Hours.*

Liver in per cent of body weight.	Glycogen content per 100 gm. of liver.
	<i>gm.</i>
2.63	0.421
2.72	0.264
2.66	0.498
2.67	0.340
2.44	0.270
2.36	0.512
2.69	0.478
Average.....2.59	0.397

The glycogen values of Tables II to IV are represented graphically in Fig. 1. The curve illustrating the rate of glycogen formation from glucose is S-shaped. The rate increases gradually up to the 2nd hour, reaches its maximum between the 2nd and 3rd hour and diminishes again in the following hour. Between the 4th and 5th hour no glycogen is deposited, in spite of the fact that the absorption of sugar from the intestine proceeds at an undiminished rate. A definite glycogen maximum of the liver has been reached. The curve for fructose follows a straight line between the 2nd and 4th hour of absorption. In the following hour the amount of glycogen deposited in the liver is very small. The curve for galactose is flat, indicating that this sugar is very slowly converted into liver glycogen. A comparison of the curves

shows that glucose and fructose are on a par as glycogen formers. This is very remarkable, since the former sugar is absorbed twice as fast as the latter. Galactose, on the other hand, which is absorbed at about the same rate as glucose, plays an unimportant rôle as a source of liver glycogen. This is not due to a low permeability of the liver cells for this sugar, since in former experiments (2) all three sugars were found to permeate with equal rapidity into the liver cells.

TABLE II.

*Rate of Glycogen Formation in Liver of Rat during Absorption of Glucose from the Intestine. A, After Giving Glucose Alone. B, After Giving Glucose plus Insulin (15 Units Per 100 Gm. of Body Weight). Each Figure Is an Average of Five to Six Experiments.*

A. Glucose alone.					B. Glucose plus insulin.							Length of absorption period.
Liver in per cent of body weight.	Absorption coefficient.*	Blood sugar.	Glycogen formed per 100 gm. of liver.	Percentage of sugar absorbed that is deposited as liver glycogen.	Liver in per cent of body weight.	Absorption coefficient.*	Blood sugar.	Glycogen formed per 100 gm. of liver.	Percentage of sugar absorbed that is deposited as liver glycogen.	hrs.		
											gm.	
2.96	0.183	169	0.38±0.11	6.1	3.00	0.169	73	0	0	1		
2.88	0.188	201	0.91±0.27	7.0	3.09	0.180	68	0.71 ±0.11	6.1	2		
3.13	0.176	189	2.66±0.49	14.7	2.89	0.175	60	0.73 ±0.20	4.0	3		
3.22	0.176	178	3.68±0.53	16.8	3.06	0.169	70	1.14 ±0.39	5.2	4		
3.24	0.175	173	3.65±0.52	13.5	3.20	0.189	71	1.77 ±0.29	6.0	5		

\* The absorption coefficient is the amount of sugar absorbed per 100 gm. of body weight per hour.

The curves for glucose plus insulin and fructose plus insulin reveal how profoundly the glycogen formation in the liver is inhibited by an excess of the pancreatic hormone. The inhibiting influence is stronger in the case of fructose than in the case of glucose. In the first 3 hours of fructose absorption no liver glycogen was formed, while after 4 and 5 hours a small amount was deposited. During the absorption of glucose, glycogen was formed in the 2nd hour and this could not be prevented, even if

the largest doses of insulin were injected. It should be noted that the insulinized animals absorbed nearly the same amount of sugar as the normal animals and that hypoglycemic symptoms were absent. The significance of the lessened deposition of glycogen in the liver of insulinized animals has been discussed in the preceding paper and need, therefore, not be repeated here. When only 1 unit instead of 15 units was injected, the rate of glycogen formation from glucose was only slightly inhibited, as

TABLE III.

*Rate of Glycogen Formation in Liver of Rat during Absorption of Fructose from the Intestine. A, After Giving Fructose Alone. B, After Giving Fructose plus Insulin (15 Units per 100 Gm. of Body Weight). Each Figure is an Average of Four to Five Experiments.*

A. Fructose alone.				B. Fructose plus insulin.					Length of absorption period.
Liver in per cent of body weight.	Absorption coefficient.*	Glycogen formed per 100 gm. of liver.	Percentage of sugar absorbed that is deposited as liver glycogen.	Liver in per cent of body weight.	Absorption coefficient.*	Blood sugar.	Glycogen formed per 100 gm. of liver.	Percentage of sugar absorbed that is deposited as liver glycogen.	
	gm.	gm.			gm.	mg.	gm.		hrs.
2.89	0.076	0.48 ± 0.23	18.2	2.82	0.081	75	0	0	1
2.97	0.070	1.60 ± 0.28	34.0	2.78	0.075	49	0	0	2
2.96	0.077	2.79 ± 0.46	35.8	2.72	0.076	54	0	0	3
3.23	0.081	3.95 ± 0.39	39.4	2.82	0.080	66	0.70 ± 0.21	6.2	4
3.11	0.079	4.23 ± 0.51	33.3	3.06	0.075	93	0.79 ± 0.29	6.4	5

\* The absorption coefficient is the amount of sugar absorbed per 100 gm. of body weight per hour.

is shown in Table V. In previous experiments (3) on the liver of sugar-fed and insulinized rabbits, a glycogen deposition of about the same rate as in control animals was observed. The insulin dose in these experiments was apparently too small to show the strong inhibiting effect.

Table IV shows that the percentage of the absorbed galactose that is excreted in the urine increases with increasing length of absorption, in spite of the fact that the rate of absorption remains constant. Thus in 1 hour 27 per cent appears in the urine, in 2 hours 41 per cent, in 3 hours 51 per cent, and in 4 hours 60 per

cent. The excretion of galactose in the urine shows many striking features and will be dealt with in the sixth paper of this series.

The previous observations on the mechanism of absorption of sugars from the intestine are confirmed by the present investigation. When the data of Tables II to IV are used for a graphic

TABLE IV.

*Rate of Glycogen Formation in Liver and Rate of Excretion of Sugar in Urine during Absorption of Galactose from the Intestine. Each Figure Is an Average of Four to Five Experiments.*

Liver in per cent of body weight.	Absorption coefficient.*	Percentage of sugar absorbed that is excreted.	Glycogen formed per 100 gm. of liver.	Percentage of sugar absorbed that is deposited as liver glycogen.	Percentage of sugar retained that is deposited as liver glycogen.	Length of absorption period.
	gm.		gm.			hrs.
2.98	0.184	27.4	0	0	0	1
3.02	0.179	40.9	0.52 $\pm$ 0.19	4.4	7.4	2
2.86	0.178	51.1	0.68 $\pm$ 0.22	3.6	7.3	3
3.16	0.186	60.5	1.16 $\pm$ 0.28	4.9	12.5	4

\* The absorption coefficient is the amount of sugar absorbed per 100 gm. of body weight per hour.

TABLE V.

*Rate of Glycogen Formation from Glucose After Giving 1 Unit of Insulin. (In Experiments in Tables II and III 15 Units Were Injected). Each Figure Is an Average of Four Experiments.*

Liver in per cent of body weight.	Absorption coefficient.	Blood sugar.	Glycogen formed per 100 gm. of liver.	Percentage of sugar absorbed that is deposited as liver glycogen.	Length of absorption period.
	gm.	mg.	gm.		hrs.
2.92	0.188	130	0.25 $\pm$ 0.13	3.9	1
3.04	0.172	67	0.89 $\pm$ 0.18	7.9	2

illustration of the rate of absorption, it will be found that the absorption of glucose, fructose, and galactose follows a straight line. The absorption coefficients for these three sugars were the same as previously reported, as is shown by the following summary. It will also be noted that insulin has no influence on the rate of absorption of sugars from the intestine.

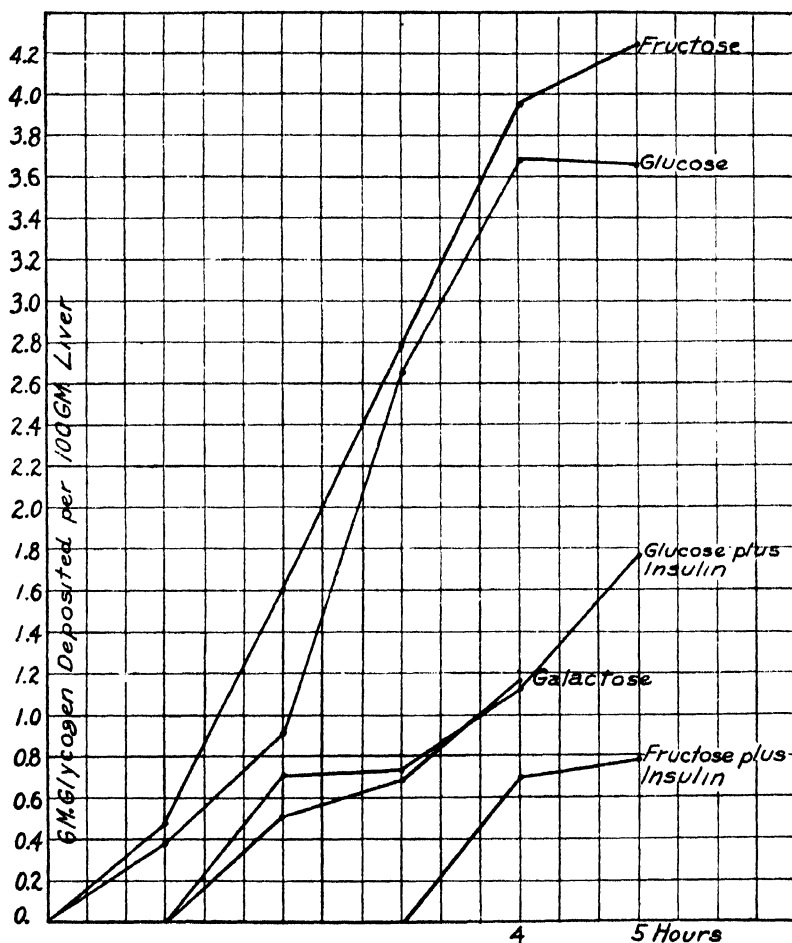


FIG. 1. The rate of glycogen formation in the liver of normal and insulinized rats.

*Average Absorption Coefficient.*

Glucose.	Fructose.	Galactose.	
0.178	0.077	0.196	Former experiments (1).
0.179	0.076	0.182	Present " (normal animals).
0.176	0.077		" " (insulinized animals).

## DISCUSSION.

In previous work on the glycogen formation in the liver quantitative data on the participation of this organ in the carbohydrate metabolism of the whole body have been lacking. The result was that the rôle of the liver has generally been overestimated. In Tables II to V the percentage of the total amount of sugar absorbed that is retained in the liver as glycogen, has been calculated. The retention of glucose reaches its maximum in 4 hours and corresponds then to 17 per cent of the amount absorbed. Even if a liberal estimate for the glucose oxidation in the liver is made, one finds that in 4 hours the liver can dispose of only one-fifth to one-fourth of the total amount of sugar metabolized in the body. Since the glycogen formation ceases after 4 hours of glucose absorption, the participation of the liver in the disposal of sugar is negligible in later periods. On the other hand, one will be prevented from committing an injustice, if one considers that the liver of rats fasted for 48 hours constitutes only 3 per cent of the body weight.

If one compares the percentage retention from glucose and from fructose, one has to take into account that the former sugar is absorbed twice as fast as the latter, while the rate of glycogen formation from both sugars is the same. The retention of fructose also reaches its maximum in 4 hours and amounts to 39 per cent of the absorbed sugar. The difference between the two sugars is still greater after 2 hours, since 34 per cent from fructose and only 7 per cent from glucose are retained as liver glycogen. Hence, the liver is of greater importance for the removal of the former sugar than for the latter. This may be the experimental explanation, why the tolerance for fructose rather than for glucose is used as a test for liver function.

The influence of insulin on liver glycogen has been discussed by the author in 1925 (4). Since that time several new papers have appeared, which seem to confuse the issue. A discussion of the pertinent data would, therefore, not seem out of place. Grevenstuck and Laqueur (5) made experiments on fasting rabbits using the abdominal window method of the author. They found in confirmation of the author's results that large doses of insulin have either no influence or lead to a decrease in the liver glycogen. Frank, Hartmann, and Nothmann (6) maintained that small



doses of insulin (0.1 unit per kilo) increase the liver glycogen of fasting rabbits, but this could not be substantiated in a later communication of Grevenstuk and Laqueur (7). The latter authors imply that, since insulin does not cause glycogenesis in the liver of fasting animals, it could not have this effect under any conditions. This is in opposition to a number of observations. Glycogen deposition in the liver under the influence of insulin has been demonstrated for fasting and phlorhizinized animals by Cori (4) and for sugar-fed and phlorhizinized animals by Nash (8). The same effect was observed by Cori (4) and by Hédon (9) on the liver of fasting and depancreatized animals and by Banting and coworkers (10) on depancreatized animals that were fed with carbohydrates. Another error of Grevenstuk and Laqueur has to be pointed out. They mention Cori's experiments on the liver of fasting and insulinized mice (16 injected mice showed, as an average, 39 per cent less liver glycogen than 16 control mice) and confront them with the experiments of Bissinger and Lesser (11), who found an increase in the glycogen content of the whole mouse following an injection of glucose plus insulin. The fallacy of such a comparison is fully explained by the data of the preceding paper. Even if no glycogen is deposited in the liver, an abundant glycogen deposition may occur in the muscles.

#### SUMMARY.

1. Curves illustrating the rate of glycogen formation in the liver of the rat during the absorption of glucose, fructose, and galactose have been presented.

2. Glucose and fructose are on a par as glycogen formers, even though the former sugar is absorbed twice as fast as the latter. Galactose plays an unimportant rôle as a source of liver glycogen.

3. After 4 hours of glucose or fructose absorption the glycogen formation in the liver ceases or is markedly diminished, indicating that a glycogen maximum has been reached.

4. Large doses of insulin almost completely suppress the glycogen formation in the liver from glucose and from fructose.

5. The rôle of the liver in the carbohydrate metabolism of the whole body could be measured quantitatively by calculating the percentage of the total amount of sugar absorbed that is retained as liver glycogen. The maximum retention occurred in 4 hours

and amounted to 17 per cent in the case of glucose and to 39 per cent in the case of fructose.

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## POTASSIUM IN ANIMAL NUTRITION.

### IV. POTASSIUM REQUIREMENTS FOR NORMAL GROWTH AND MAINTENANCE.

By HARRY G. MILLER.

*(From the Department of Agricultural Chemistry, Oregon Experiment Station, Corvallis.)*

(Received for publication, August 11, 1926.)

Marked retardation in the growth of young rats on a ration containing 0.1 per cent potassium was reported in a previous publication (1). Based upon experimental data the minimum potassium requirement was stated to be between 0.55 and 1.44 gm. per kilo of ration. Obviously this does not state the minimum daily potassium requirements for growth. The potassium intake would depend on the ration consumed and the latter would depend as we have observed on the potassium content of the ration. In the work reported here the potassium intake has been controlled by adding a potassium citrate solution of known potassium content to the minimum amount of food consumed by the animal. Definite quantities of ration were further added as eaten by the animal. In this way the daily allowance of potassium was all consumed and the animals were given access to all the ration they desired.

The ration used was composed of casein 18, dextrin 74, agar 2, cod liver oil 2, salt mixture 4, and 3 cc. of yeast extract a day per animal. The casein was a commercial casein that had been washed with distilled water acidified with acetic acid. The dextrin was made from corn-starch which had been washed with acidified distilled water. The yeast extract was prepared as described before (1). A modification in the method was tried by extracting the yeast with a 50 per cent alcoholic solution containing tartaric acid and removing the alcohol as formerly by distillation under reduced pressure. The extract, however, contained appreciable quantities of potassium and could not be used. Extracting the yeast with water and concentrating the extract on the steam bath

seemed to be a necessary step in the removal of potassium. The potassium content of this ration with the potassium-free salt mixtures contained less than 0.01 per cent potassium. The compositions of the salt mixtures used here are given in a former publication (2). The animals were kept in wire cages and not allowed access to shavings or paper litter.

All animals failed to grow on this minimum potassium ration but responded to the addition of the potassium-containing salt

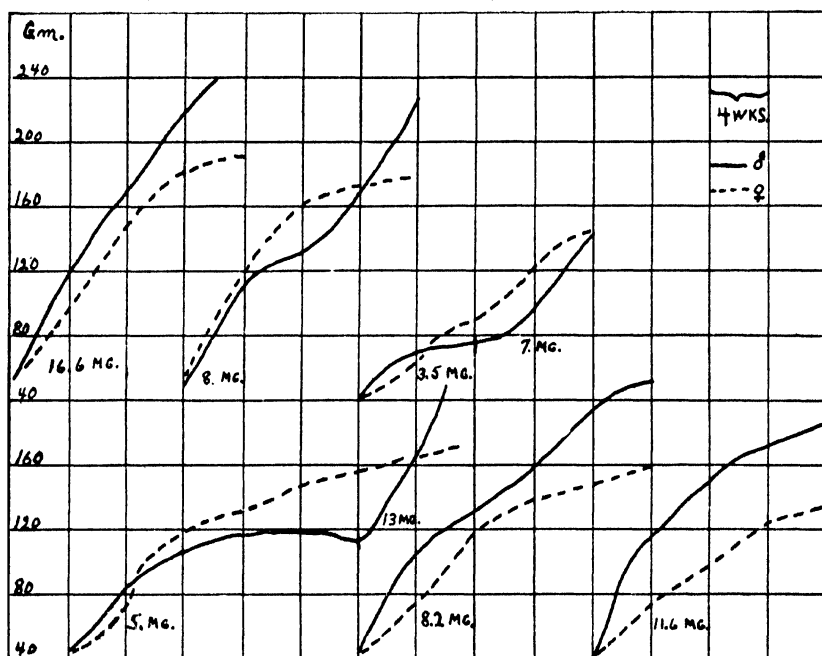


CHART I.

mixture or potassium citrate in the drinking water or in the feed. This was demonstrated with many animals, thus satisfactorily confirming that potassium was the limiting factor and that an animal cannot grow without potassium in the diet.

Information as to minimum potassium requirements for growth may be obtained by examining the growth curves in Chart I. These curves are representative of animals receiving the daily quantity of potassium as indicated in mg. under the curves. The dotted lines and continuous lines represent the growth of the fe-

males and the males respectively. The growth curve of the male animal receiving 16.6 mg. of potassium agrees with the growth curve of male animals receiving the identical ration with a complete salt mixture. As the daily quantity of potassium becomes less the growth curve ascends at a lower rate. This is observed with the animal receiving 11.6 mg. of potassium. With the female 8.0 mg. of potassium suffice for normal growth. One thing especially noticeable is that as the potassium intake becomes 8.0

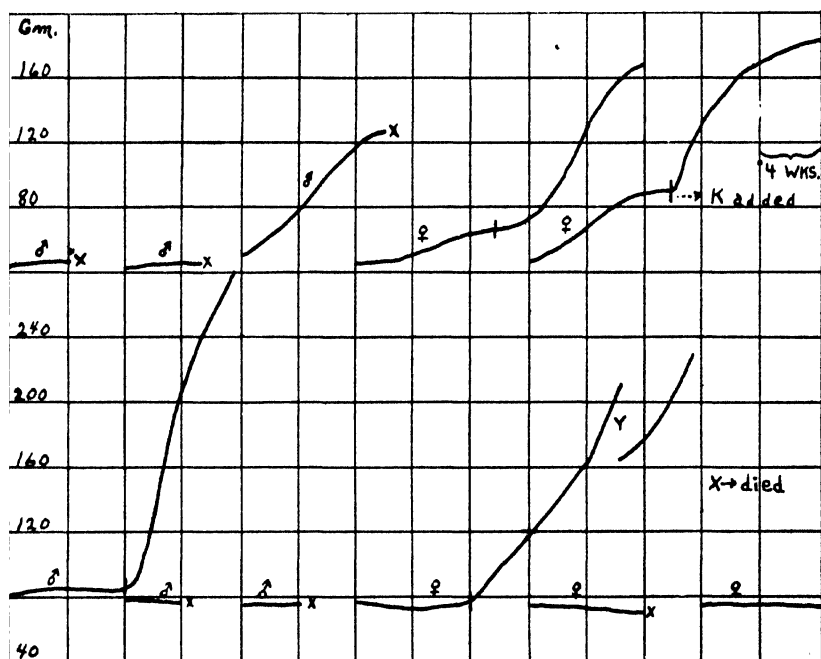


CHART II.

mg. or lower there is a tendency for the female to increase in body weight beyond that of the male of the same age and litter. This is in part explained by the greater food consumption of the female where the potassium intake is low. However, the tendency is for food consumption to decrease as the potassium intake is lowered with both sexes. Another difference in the reaction of the male and female towards minimum potassium diets is that the male invariably dies before the female (Chart II). For normal growth the male would naturally require more potassium daily

than the female due to the more rapid increase in body weight. Apparently the male also requires more potassium in maintaining its bodily functions other than growth inasmuch as it succumbs first on the minimum potassium ration while maintaining the same body weight as the female and also grows at a slower rate on low potassium intake. A study of the excretion of potassium and the potassium content of males and females of equal body weight and age will give further information on these phenomena. No permanent injury occurs in either sex if the animal is deprived of potassium for a limited period of time.

Formerly the writer (1) reported his observation on a group of animals that received a low potassium diet for 4 weeks. They responded to an increase of potassium in their ration but later developed an eye infection and died. In repeating this experiment no pathological symptoms have appeared; in fact, all animals responding to potassium have developed normally and in some cases reproduction has occurred. The writer's previous statement that inadequate potassium during early growth may effect a permanent injury (1) will have to be withdrawn unless the animal is maintained on a minimum potassium diet for a time so long that it will not respond to an increased potassium intake. It was unusual for an animal to live more than 8 weeks on the minimum potassium diet. In a previous investigation (1) only one animal out of four receiving increased potassium after 10 weeks on the minimum potassium diet manifested any inclination to increase in body weight. Chart II illustrates the behavior of animals on the minimum potassium diet and their response to potassium citrate in the diet. After an 8 week period there was an immediate response. Of the two females that survived 10 weeks, one responded and the other with difficulty, following the introduction of potassium in the diet.

Mature animals that have developed on a synthetic ration will maintain themselves on low potassium rations (2.0 mg. daily). Quantitative data on the excretion of potassium show that there is no retention of potassium. Information on the minimum requirements for maintenance will have to be determined during later investigations.

## CONCLUSIONS.

1. Young rats fail to grow and eventually die within 8 weeks when the daily intake of potassium is 1.0 mg. or less.

2. The minimum daily potassium requirement for normal growth in the male is approximately 15.0 mg.; for the female it is approximately 8.0 mg.

3. The male apparently requires more potassium than the female for purposes other than increase in body weight.

4. The daily maintenance requirement of a mature animal for potassium is not over 2.0 mg.

5. Like certain other nutrients, continual removal of potassium from the diet may lead to the stage where the animal no longer responds to potassium.

6. Based upon the potassium requirements of this type of animal and the content of potassium in natural foodstuffs, the potassium requirements for animal development are abundantly satisfied in the ordinary ration.

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## POTASSIUM IN ANIMAL NUTRITION.

### V. INFLUENCE OF POTASSIUM ON URINARY AND FECAL EXCRETION OF SODIUM, CHLORINE, CALCIUM, AND PHOSPHORUS.

By HARRY G. MILLER.

*(From the Department of Agricultural Chemistry, Oregon Experiment Station, Corvallis.)*

(Received for publication, August 11, 1926.)

A temporary increase in the urinary sodium and chlorine excretion of the growing pig caused by a marked increase in the potassium intake was reported by the writer (1). This study was later extended to observe the effect of high potassium intake on the total excretion of sodium, chlorine, phosphorus, and calcium of the mature rat (2). Here also there was an immediate increase in the sodium and chlorine excretion with the sodium excretion remaining greater throughout the period of increased potassium intake. Suggestions for further investigation which arose from this work were as to whether the urinary excretion of sodium and chlorine represents the loss of the body supply of these elements and if the growing animal reacts in the same way as the mature animal does when the ration is supplemented with potassium salts.

Two growing and one mature male rats were used in this work; each one received a different ration and the distribution of the mineral excretion between the urine and feces was determined during low and high potassium intake. The method used for separating the urine and feces was one the writer observed in use at the University of California laboratory, Berkeley. The analytical methods were the same as used previously (2) except the sodium and potassium were weighed as sulfates and the potassium chloroplatinate washed with 10 per cent ammonium chloride solution saturated with potassium chloroplatinate.

The daily quantity of feed was added in three or four portions to the porcelain dish in order to avoid scattering. Potassium

salts, when added, were added in solution form directly to the milk. 1 cc. of the potassium salt solutions contained 0.1 gm. of potassium. The sodium citrate solution contained the equivalent of sodium or 0.059 gm. per cc.

The average daily mineral excretions shown in Tables I, II, and III are the average over a 4 day period in Tables II and III and

TABLE I.  
*Average Daily Mineral Excretion. Growing Animal.*

Daily food intake.					Urine.					Feces.				
Ration.*	Milk.	Potassium chloride solution.	Potassium citrate solution.	Sodium chloride solution.†	Potassium.	Sodium.	Chlorine.	Phosphorus.	Calcium.	Potassium.	Sodium.	Chlorine.	Phosphorus.	Calcium
gm.	cc.	cc.	cc.	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
12.0	10.0			1.0	30.0	10.0	22.0	1.2	1.6	4.3	2.4	0.6	21.0	23.0
12.0	10.0			1.0	33.0	10.0	22.0	1.7	1.6	5.3	1.9	0.4	22.0	25.0
12.0	15.0		1.5	1.0	170.0	33.0	28.0	8.8	2.6	8.7	0.9	0.3	23.0	33.0
12.0	15.0		1.5	1.0	162.0	25.0	27.0	4.6	3.1	10.2	1.0	0.4	24.0	31.0
12.0	20.0		2.0	1.5	230.0	58.0	35.0	9.0	2.9	4.5	2.6	0.3	23.0	30.0
12.0	20.0			1.5	55.0	20.0	41.0	4.0	2.3	3.9	1.0	0.3	17.0	27.0
10.5	20.0			1.5	30.0	8.7	30.0	3.0	2.2	5.2	1.8		27.0	36.0
10.6	20.0		2.0	1.5	217.0	58.0	41.0	12.3	2.7	5.7	2.3		19.0	33.0
10.6	20.0		2.0	1.5	206.0	43.0	31.0	8.7	2.5	5.4	1.4		27.0	44.0
10.5	20.0		2.0	1.5	273.0	55.0	35.0	11.0	3.8	5.7	1.2		21.0	35.0
9.0	20.0		2.0	1.5	218.0	51.0	31.0	10.6	2.0	7.3	2.3		22.0	38.0
8.0	20.0			1.5	44.0	18.0	33.0	5.8	2.3	7.0	4.6		27.0	42.0
8.0	20.0			1.5	53.0	23.0	37.0	11.3	1.7	5.6	4.6		26.0	42.0
8.0	20.0	2.0		1.5	218.0	52.0	210.0	12.0	1.9	6.3	2.3		23.0	38.0
8.0	20.0	2.0		1.5	218.0	44.0	186.0	9.2	1.7	11.1	3.0		26.0	44.0
8.0	20.0	2.0		1.5	217.0	47.0	204.0	7.0	1.8	9.0			33.0	54.0

\* Corn 87, casein 10, yeast 1,  $\text{CaCO}_3$  1, cod liver oil 1.

† 1.0 gm. of NaCl in 50.0 cc.

for a 3 day period in Table I. The chlorine excretion by way of the intestine, as shown in Table I, is less than 1.0 mg. per day, which averages about 2 per cent of the total. Due to the small proportion of chlorine in the feces the chlorine determination was confined only to the urine.

The data on the two growing animals are given in Tables I and II and Table III gives the data obtained with the mature

animal. The chlorine excretion, as reported before, rose temporarily after potassium increase in the diet. The increased sodium excretion during the high potassium period is characteristic of all animals and periods. This occurs while the animals are increasing normally in weight.

The animal in Table I increased from 140 to 240 gm. during 30 days and the animal in Table II increased 65 gm. in weight during the 28 days in the metabolism cage. Two other males from the same litter were placed on screen and given the same ration plus milk as in Table II. One of these animals was given in addition 150 mg. of potassium daily. All three of these animals

TABLE II.  
*Average Daily Mineral Excretion. Growing Animal.*

Daily food intake.			Urine.					Feces.			
Ration.*	Milk.	Potassium citrate solution	Potassium.	Sodium.	Chlorine.	Phosphorus.	Calcium.	Potassium.	Sodium.	Phosphorus	Calcium.
gm.	cc.	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
12.0	10.0		4.5	14.2	15.0	13.6	0.7			13.2	13.5
12.0	10.0		7.5	14.0	17.0	14.7	1.2			11.2	10.3
12.0	10.0	1.0	98.5	28.0	20.0	17.0	1.2	2.3	1.4	12.8	13.5
12.0	10.0	1.0	96.2	20.7	12.0	15.4	1.0	3.6	1.2	8.8	6.9
12.0	15.0	2.0	191.0	17.0	18.0	14.3	1.9	9.2	1.4	7.0	7.9
12.0	20.0	2.0	177.0	31.0	18.0	12.1	2.5	8.4	2.6	9.7	8.4
12.0	20.0		19.7	15.0	21.0	15.4	2.5	3.8	3.5	13.7	12.1

\* Dextrin 74, casein 20, yeast 2, agar 2, Salt B 2.

grew at the same rate while receiving different levels of potassium. No apparent retardation of growth resulted from consuming these large quantities of potassium but where observations were made there was a depletion of the body supply of sodium. This was more pronounced with the young animals than with the mature animal; however, the animal in Table III weighed 340 gm. which lowered the ratio of potassium to body weight and undoubtedly made its action less effective.

The amounts of sodium, chlorine, and potassium excreted in the urine represented by far the larger proportion of the total excretion

of these elements. Regardless of the nature of the ration the total daily quantities of chlorine, sodium, and potassium excreted

TABLE III  
*Average Daily Mineral Excretion. Mature Animal.*

Daily food intake.					Urine.					Feces.			
Ration.*	Milk.	Potassium citrate solution.	Potassium chloride solution.	Sodium citrate solution.	Potassium.	Sodium.	Chlorine.	Phosphorus.	Calcium.	Potassium.	Sodium.	Phosphorus.	Calcium.
gm.	cc.	cc.	cc.	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
12.0	20.0				16.4	7.5	14.5	10.0	1.6			18.2	22.7
12.0	20.0				19.0	5.7	14.0	10.0	1.4	1.5	0.9	17.8	23.9
12.0	20.0				21.5	9.2	16.5	8.5	1.1	3.0	1.6	18.0	24.6
12.0	20.0	2.0			209.0	12.0	21.0	19.0	2.7	7.1	1.0	16.0	25.2
12.0	20.0	2.0			195.0	23.0	16.5	16.5	3.7	12.5	1.5	15.0	24.6
12.0	20.0	2.0			210.0	5.0	16.5	16.0	3.4	3.0	0.8	12.5	23.7
12.0	20.0				20.0	6.5	15.0	9.0	2.0	4.0	1.6	15.5	23.0
12.0	20.0				22.0	7.8	17.5	9.0	1.8	1.0	1.0	14.0	19.0
12.0	20.0				21.0	8.2	17.5	10.0	2.0	1.3	0.5	18.0	29.0
12.0	20.0		2.0		191.0	14.2	187.0	14.5	2.4	0.4	0.8	14.0	21.0
12.0	20.0		2.0		198.0	7.2	189.0	12.0	2.4	0.3	0.4	17.0	24.0
12.0	20.0		2.0		202.0	15.0	194.0	16.0	2.9	0.3	0.5	22.0	31.5
12.0	20.0		2.0		211.0	5.4	201.0	13.0	2.2	8.0	2.4	21.0	29.0
12.0	20.0		2.0		25.0	12.0	41.0	17.0	2.0	1.6	1.8	23.0	30.5
12.0	20.0				21.5	9.5	17.0	12.0	1.5			12.0	26.8
12.0	20.0				26.0	8.0	20.0	17.0	1.7			17.0	26.8
12.0	20.0	2.0			212.0	12.0	15.7	17.0	2.6			14.4	28.0
12.0	20.0	2.0			205.0	15.0	15.0		2.5			14.3	27.5
12.0	20.0				24.0	9.0	17.0	13.5	2.2			15.0	24.5
12.0	20.0				25.0	9.0	17.0	11.8	1.4			15.6	27.0
12.0	20.0				26.0	12.0	20.0	15.1	1.5			18.7	30.2
12.0	20.0	2.0			201.0	12.0	20.0	15.0	2.1			19.2	32.5
12.0	20.0	2.0			189.0	22.0	17.0	13.0	3.1			19.0	34.8
12.0	20.0				42.0	4.5	15.0	10.8	1.4			19.5	31.2
12.0	20.0				24.0	9.0	17.0	9.1	1.7			20.7	31.7
12.0	20.0			2.0	38.0	136.0	20.0	14.1	2.7			13.5	23.5
12.0	20.0			2.0	26.0	142.0	21.0	13.0	3.0			13.6	25.0
12.0	20.0			2.0	34.0	166.0	24.0	16.5	3.3			14.6	26.0

\* Dextrin 84, casein 10, agar 4, yeast 2.

in the feces remained approximately the same. While there was some variation it does not account for the increased urinary sodium

and chlorine excretion caused by potassium assimilation. The daily excretion of calcium in the urine was about the same regardless of the intake, but the proportion and quantity of phosphorus in the urine and feces varied according to the nature of diet. In Table II, where sodium phosphate was in the salt mixture, over 50 per cent of the phosphorus was excreted by way of the kidney.

Where potassium caused an increase in urinary phosphate there was a decrease in the fecal excretion. High potassium intake did not cause an increase in the total calcium and phosphorus excretion. In fact, the excretion of these elements decreased during active growth of the animal while the intake of potassium was high. From these results, a quantitative record of the urinary excretion of sodium, chlorine, and potassium gives the information pertaining to the loss of these elements from the body. With phosphorus, both the urinary and fecal excretion must be considered, while with calcium over 95 per cent is excreted by way of the intestine. The action of potassium on the excretion of other elements appeared to be the same whether it was given as potassium citrate or potassium chloride. Sodium citrate apparently increased the urinary phosphorus excretion but its feeding was not extensive enough to state what effect it had on the excretion of other elements.

In the data reported here the excretion of sodium during high potassium consumption was greater than the intake of sodium. At the same time the animal increased in weight. Apparently this can be interpreted in no other way except that the percentage of sodium in the body has decreased. Any serious condition resulting would have to be demonstrated by feeding potassium over a longer period and a histological examination of the tissue. One thing to consider in this work is that potassium was fed at an abnormally high level. 200 mg. of potassium daily is about ten times the amount necessary for normal growth and three to four times that in a ration of natural foodstuffs.

#### CONCLUSIONS.

1. The increased excretion of sodium and chlorine caused by increasing the potassium intake of the rat is found entirely in the urinary content of sodium and chlorine.

2. Sodium excretion was persistently higher during the period of high potassium intake. The amounts of potassium fed were abnormally high but did not retard growth or cause a continued increased excretion of chlorine, phosphorus, and calcium.

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## NOTES ON SUGAR DETERMINATION.

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### I.

#### *Influence of Alkalinity on Copper Reduction.*

In the course of the past few years we have had the experience that whenever acid-glucose solutions—as represented for instance by hydrolysates of glycogen—were neutralized with sodium carbonate or bicarbonate, the values obtained by copper reduction have been somewhat inconsistent. On closer investigation upon pure glucose solutions it has been found that added bicarbonate, even in slight amounts, causes appreciable increase of the copper reduction values. This is in line with the well known fact that a lowering of the alkalinity of copper solutions entails an increase in reduction values. Shaffer and Hartmann (1) observed an increase of 10 per cent with their carbonate-citrate copper reagent as compared with the reduction given by the more alkaline Fehling solution. It is in accord also with the observation of the same authors that when they have substituted Rochelle salt for tartaric acid in their carbonate-tartaric acid copper reagent the resulting solution had about 10 per cent lower reduction values. This difference is probably due to the bicarbonate formed (and consequent decrease of alkalinity) when the free tartaric acid is neutralized by sodium carbonate.

These facts prompted us to determine more accurately the effect of varying alkalinity upon the reduction values in the system, glucose-alkaline copper solution. To this end a series of nine copper reagents was prepared in all of which Rochelle salt was used instead of tartaric acid; the reagent of highest alkalinity contained 40 gm. of sodium carbonate per liter, the other eight solutions were



made up with buffer mixtures containing varying amounts of sodium carbonate and bicarbonate. The rest of the components were identical with those of the original Shaffer-Hartmann reagent. The reduction values of the nine reagents were determined on pure glucose solutions of concentrations of 0.04, 0.03, 0.01, and 0.00252 per cent, following the standard procedure, the actual amounts of glucose in the 5 cc. portions used for each determination being 2, 1.5, 0.5, and 0.126 mg. respectively.

In Table I are presented the sodium carbonate-bicarbonate ratios of the several reagents and the respective reduction values,

TABLE I.

*Reduction Values of Shaffer-Hartmann Reagents with Varying Amounts of Carbonate and Bicarbonate.*

No. of reagent.	Molar concentration of:		Apparent pH of Na <sub>2</sub> CO <sub>3</sub> mixture.	Cc. of iodine consumed by				Mg. of Cu reduced by			
	NaHCO <sub>3</sub>	Na <sub>2</sub> CO <sub>3</sub>		2.0	1.5	0.5	0.126	2.0	1.5	0.5	0.126
				mg. of glucose.				mg. of glucose.			
I	0.400	0.100	9.45	15.60	11.07	3.02		4.96	3.52	0.95	
II	0.375	0.125	9.55	16.40	11.35	3.10	0.22	5.22	3.60	0.99	0.07
III	0.350	0.150	9.68	16.80	12.03	3.25	0.32	5.34	3.83	1.03	0.101
IV	0.325	0.175	9.80	17.0	12.45	3.45	0.34	5.40	3.96	1.10	0.108
V	0.300	0.200	9.90	17.0	12.45	3.45	0.36	5.40	3.96	1.10	0.115
VI	0.275	0.225	10.00	16.40	12.08	3.40	0.39	5.22	3.84	1.08	0.117
VII	0.250	0.250	10.10	16.0	11.90	3.13	0.27	5.09	3.79	1.00	0.086
VIII	0.100	0.350	10.60	14.3	10.6	2.83	0.23	4.55	3.37	0.90	0.075
IX	0	0.400	11.60	12.35	9.4	2.50	0.24	3.93	2.99	0.80	0.076

expressed in quantities of 0.005 N iodine consumed in reoxidation of the cuprous oxide formed.

A word has to be said regarding the pH values in the table: these are by no means actual pH values of our copper reagents, not even those of our carbonate-bicarbonate buffer mixtures but are cited from the data by Auerbach and Pick (2) on sodium carbonate-bicarbonate mixtures of the same ratios but lower concentration (0.2 molar solutions). We have not attempted to determine the actual pH for optimum copper reduction by glucose. This would not be accomplished even if the pH values of the reagents were accurately known, as presumably a considerable

shift in reaction occurs during the 15 minutes warming, due to acid formation from the sugar oxidation, and from the loss of carbonic acid from the bicarbonate. However, the order of *actual differences* between the pH values of the individual reagents

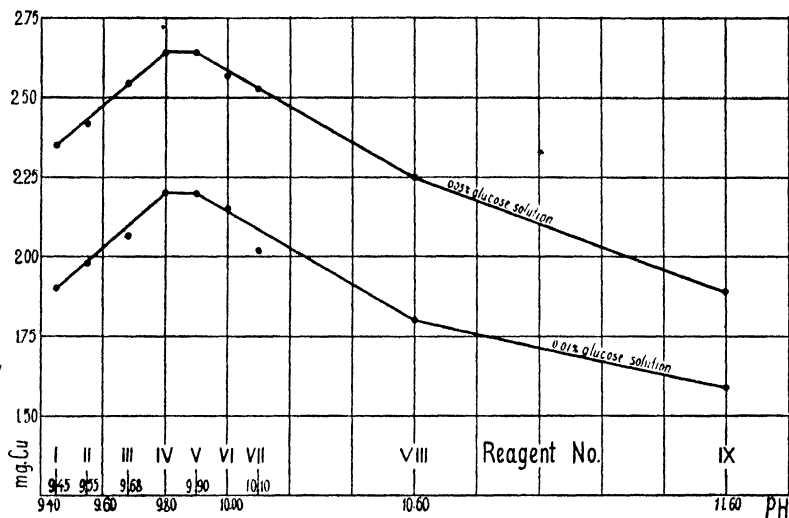


FIG. 1.

in our series is indicated by the figures cited, and this will suffice to emphasize the fact that *comparatively small changes in alkalinity produce considerable differences in reduction values*.<sup>1</sup>

<sup>1</sup> After this paper had been sent to press, Visscher (3) reported his observation that the addition of acid phosphate to sugar solutions in determinations by the Shaffer-Hartmann macro method effected a lowering of the reduction values by about 20 per cent as compared with results obtained on the addition of a similar amount of dibasic phosphate. Since the total concentration of the phosphate in Visscher's experiments is constant apparently the shift in hydrogen ion concentration alone is responsible for the change in reduction values. I am emphasizing in my present paper that for the determinations by alkaline copper reagents in general the sugar solutions ought to be neutral or at least approximately so, and must not possess sufficient buffer value to change materially the alkalinity of the copper reagent. Where such buffer action is unavoidable it is necessary to determine anew the reducing value of the copper reagent in the presence of the buffer, as done by Visscher, instead of using the published tables of copper:sugar values.

As shown in the table, Reagents IV and V—with  $\frac{[\text{Na}_2\text{CO}_3]}{[\text{NaHCO}_3]}$  ratios  $\frac{7}{13}$  and  $\frac{2}{3}$  respectively—*furnish the highest reduction values.*

(In our electrometric measurements we have found for the carbonate-bicarbonate buffer mixture, No. IV, pH = 9.40, for No. V, pH = 9.55.) Departure in either direction from this quite narrow range of optimum alkalinity causes a decline of the reduction values, the change being relatively greater in the direction of increasing alkalinity.

For better illumination of these relations two curves are given in Fig. 1 showing the alterations of reduction values with changing alkalinity. The curves are based on data in Table I. Against the "apparent" pH values on the abscissa are plotted on the ordinate corresponding values of the ratio  $\frac{\text{copper reduced}}{\text{glucose}} = \text{mg. of copper reduced by 1 mg. of glucose.}$  From the fairly close parallelism of the two curves it can be seen that the effect of variations in alkalinity is much the same for different concentrations of glucose, regardless of the well known fact that the ratio:  $\frac{\text{copper reduced}}{\text{glucose}}$  suffers a rapid decline with decreasing concentration of sugar.

The question arises here, in what manner is the process of oxidation-reduction in this system affected by the degree of alkalinity: by an alteration in the condition of the copper complex, or in that of the glucose, or perhaps both. Two experimental facts seem to indicate that the variations are to be ascribed solely to changes in the reactions of glucose with changing alkalinity. First, we have found that the reduction values are subject to variations of the same nature and the same order if iron is taking the place of oxidizing agent, as in the Hagedorn-Jensen method. A second evidence is furnished by the behavior of alkaline copper solutions in which sodium citrate is substituted for Rochelle salt. Such reagents exhibit markedly depressed reduction values as compared with tartrate reagents, all other conditions in both being the same. Shaffer and Hartmann (1) found 10 per cent lower reduction with citrate in place of tartrate, and recently Folin (4) emphasizes this phenomenon speaking of "the antireducing effect of citrates" brought about by "a powerful depressive action

on the oxidative properties of dissolved copper compounds." In our present experiments we have found as much as 33 per cent drop in reduction at optimum alkalinity if citrate be substituted for Rochelle salt, and we agree with Folin that this is probably due to a change in the condition of the copper complex. But concurrently we have found that through variations of alkalinity, the reduction values of citrate reagents undergo changes in the same direction and of the same relative magnitude as those of tartrate reagents.

Thus we are confronted by two factors of distinctly different character, both altering the reduction values in the system: glucose-alkaline copper solution. The powerful depressing effect of citric acid is apparently due to a diminished oxidative intensity of the copper complex. The degree of alkalinity, on the other hand, seems to exert its effect upon reduction values by influencing the reactions of the sugar alone.

The curves in Fig. 1 might be interpreted as representing the resultant of two types of reactions in alkaline glucose solutions: one is to produce oxidizable fragments of the glucose molecule, the other to counteract the oxidation—by intramolecular oxidations, condensations, etc., between the fragments—before their oxidation is accomplished. Both these reactions are accelerated in speed by increasing alkalinity but there is a range of pH—represented by the short ridge of the curves—which favors the first of the two types of competitive reactions more than the second. Further increase of alkalinity then accelerates the velocity of the reactions of the second type which thus gradually get the upper hand and lead to diminished reduction values.

## II.

### *Modified Shaffer-Hartmann Reagent.*

The bearing of the foregoing upon the methods of sugar determinations by copper-reduction methods in general is obvious. In the light of it we understand why Folin (4) found it necessary to neutralize his tungstate filtrates for blood sugar determinations when using his modified copper reagent; and we obtain an explanation for the findings by Duggan and Scott (5) who have observed that the reduction values obtained by them with the Shaffer-

Hartmann method were slightly but definitely lower than those given by the authors of the method. In this laboratory, too, it had been noticed during the past few years that various batches of the Shaffer-Hartmann carbonate-tartaric acid reagent showed in their reduction values deviations ranging from 2 to 5 per cent. While no particular importance was attributed to this for ordinary clinical work, every new preparation has been checked with pure glucose before use in investigations.

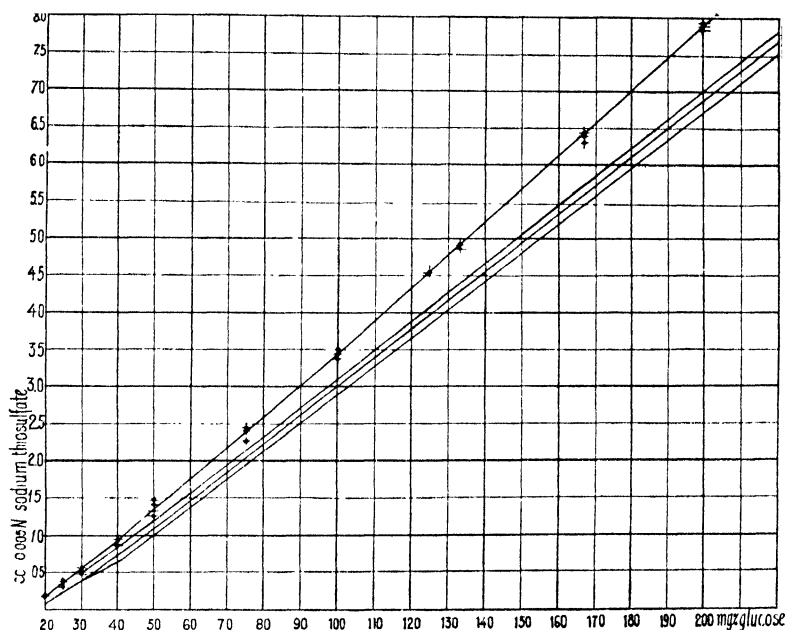


FIG. 2.

Now it is evident that these variations result from slight differences in the alkalinity of the copper reagents originating from the mode of their preparation. Namely, when, according to the directions, a solution of tartaric acid and copper sulfate is poured into a solution of sodium carbonate, carbonic acid is generated giving rise to the formation of *sodium bicarbonate* but *in every case allowing a part of the carbonic acid to escape*. The quantity lost will vary according to the temperature of the alkaline and acid solutions when poured together, and will be influenced

by the method of stirring and possibly by other small factors. This can readily be seen if we prepare two carbonate-tartaric acid reagents with the sole difference in the procedure that in one of them the carbonate and acid are united at room temperature while in the other a warm solution of tartaric acid is poured into a warm solution of sodium carbonate. In Fig. 2, Curve II represents the reduction values for the reagent prepared at room temperatures (which agree perfectly with the data by Shaffer and Hartmann, except for sugar solutions of concentrations below 8.3 mg. per cent); Curve IV is plotted from reduction values of the reagent prepared at higher temperature. The lower reduction values of the latter are obviously due to a greater loss of carbonic acid and—as a result of it—higher alkalinity. Curve III is plotted from the data of Duggan and Scott; it permits the inference that in the preparation of their reagent they have lost less carbonic acid than we in our preparation with warm solutions, however more than in our sample made up at room temperature.

It is obvious that, in order to rule out these variations in alkalinity, one has to eliminate the indirect formation of bicarbonate by using Rochelle salt instead of free tartaric acid, and adjusting the alkalinity by direct addition of sodium bicarbonate. A glance at Table I and Fig. 1 will indicate the proper choice of the  $\frac{[\text{Na}_2\text{CO}_3]}{[\text{NaHCO}_3]}$

ratio. It is evident without much comment that Solution V, in which the ratio is  $\frac{2}{3}$ , best responds to our requirements. With

this reagent the reduction is about 10 to 15 per cent greater than with the Shaffer-Hartmann reagents, as shown by Curve I in Fig. 2. Not only is the alkalinity of the modified reagent in the range of maximum reduction, but due to its buffer action this high reduction is maintained when slightly acid sugar solutions are added to it. An equal volume of a 0.025 *N* acid would shift its alkalinity only to that of Reagent IV which possesses the same reduction value; and this amount of added acid is  $12\frac{1}{2}$  times that of the Folin-Wu blood filtrate. (Folin found that 10 cc. of filtrate require 0.2 cc. of 0.1 *N* alkali for neutralization to phenolphthalein, or are equivalent to 0.002 *N* acid.)

The composition of the modified tartrate-carbonate copper reagent is as follows:

Final concentration.		gm. per liter
0.026 M	Copper sulfate (crystalline).....	6.5
0.06 "	Rochelle salt.....	12
0.2 "	Sodium carbonate (anhydrous).....	20
0.3 "	" bicarbonate.....	25
0.023 N I <sub>2</sub>	{ Potassium iodide.....	10
	" iodate.....	0.80
0.1 M	" oxalate.....	18

Dissolve the Rochelle salt, sodium carbonate, and sodium bicarbonate in about 500 cc. of water, and into this pour with stirring the copper sulfate dissolved in about 100 cc. of water; then add the solution of the other constituents and dilute to 1 liter. (Only the potassium iodate has to be weighed accurately to cgm.)

This reagent, besides furnishing perfectly consistent reduction values and possessing a buffer effect for slightly acid sugar solutions, has as a third advantage,—a greater sensitiveness at the lowest concentrations of glucose and hence permits reliable determinations of blood sugar values as low as 0.020 per cent.

For the sake of convenience we give here the procedure of sugar determination, largely quoted from Shaffer and Hartmann's communication.

Measure 5 cc. of the reagent into a large test-tube (250 × 25 mm.) and add 5 cc. of the sugar solution containing not less than 0.1 mg. and not more than 2.0 mg. of glucose. Mix by gentle shaking, cover the tube with small funnel or bottle cap or glass bulb, and keep it in a boiling water bath for 15 minutes. Cool by placing in a shallow dish of water until temperature is lowered to 35–40°C. Add with agitation 1 cc. of 5 N H<sub>2</sub>SO<sub>4</sub> (or equivalent amount) and see that all Cu<sub>2</sub>O is promptly dissolved. After about 2 minutes titrate with 0.005 N sodium thiosulfate. A blank titration on 5 cc. of the reagent is determined after heating with an equal amount of water.

The difference between the blank and the titration of a determination is equivalent to the copper reduced and thus to the sugar. The corresponding amounts of sugar, in mg. per 100 cc. of blood, are given in Table II. (For its elaboration U. S. Bureau of Standards glucose was used.)

*Example.*—The blank titration on 5 cc. of the reagent was 22.65 cc. of 0.005 N thiosulfate, the titration of a determination 18.22 cc.:

22.65 — 18.22 = 4.43; Table II gives for 4.40 cc. 121 mg., for 4.50 cc. 124 mg. per cent sugar, thus the result of the determination is 122 mg. in 100 cc. of blood.

The table is calculated for the conventional 1:10 dilutions of blood. For other cases the actual amount of glucose, in the 5 cc.

TABLE II.

*Amounts of Glucose Corresponding to Titration Values when 5 Cc. of 1:10 Blood Filtrate and 5 Cc. of Copper Reagent (Modified) Are Heated in Water Bath for 15 Minutes.*

Cc. of 0.005 N thiosulfate.	Tenths of 1 cc. of 0.005 N sodium thiosulfate.									
	0	1	2	3	4	5	6	7	8	9
	Mg. of glucose in 100 cc. of blood.									
0			21	23	26	29	31	34	36	39
1	41	44	46	49	51	53	56	58	61	63
2	65	68	70	72	75	77	80	82	84	86
3	89	92	94	97	99	101	103	106	108	110
4	113	115	117	119	121	124	126	128	130	132
5	135	137	139	141	143	146	148	150	152	154
6	157	159	161	163	165	168	170	172	174	176
7	179	181	183	185	187	190	192	194	196	199
8	201	203	205	207	210	212	214	216	218	221
9	223	225	227	230	232	234	237	239	241	243
10	245	248	250	252	254	256	259	261	263	265
11	267	270	272	274	276	279	281	283	285	288
12	290	292	294	296	299	301	303	305	308	310
13	312	314	316	318	321	323	326	328	330	332
14	334	337	339	341	343	345	347	350	352	354
15	356	359	361	363	365	367	370	372	374	376
16	378	381	383	386	388	390	392	394	396	398
17.	400									

solution used for a determination, is computed by dividing the values in the table by 200. In the above example the actual amount of sugar in 5 cc. of solution is  $\frac{122}{200}$  0.61 mg.

## III.

*Determination of Sugar in 0.2 Cc. of Blood.*

"When only blood sugar values are desired, as in following blood sugar curves in testing carbohydrate tolerance, it is prefer-



able to avoid unnecessary venous puncture and to draw the blood from the tip of a finger or the ear lobe. In this way 0.2 to 0.6 cc. can readily be obtained, amounts which are ample for determination of blood sugar (and for hemoglobin, blood counts and, with special precautions, for pH). The use of the Shaffer-Hartmann method with 0.2 cc. blood is as follows.

"Measure into a small test tube 2 cc. of  $\frac{1}{15}$  normal (0.0667 N)  $\text{H}_2\text{SO}_4$ . Measure the blood in a special 0.2 cc. pipette, deliver it into the acid and rinse the pipette several times with the liquid in the test tube. Mix the contents of tube well and after a few moments add exactly 0.8 cc. 2.5 per cent sodium tungstate. Shake well, cover the tube by cork, tin foil or rubber cap and centrifugate. With a small thread or rubber band attach a small tuft of absorbent cotton over the end of a 2 cc. pipette, and with it draw up carefully 2 cc. of the clear blood filtrate from the test tube. Deliver it into another clean test tube ( $16 \times 150$  mm.), add exactly 2 cc. of the Shaffer-Hartmann reagent, mix by gentle shaking, cover the tube with a glass bulb, and heat in boiling water for 15 minutes. Cool to about  $35^\circ\text{C}$ . Add 1 cc. 2 N  $\text{H}_2\text{SO}_4$ , shake to dissolve the cuprous oxide completely, and after a minute or two titrate very carefully with 0.005 N thiosulphate in a micro burette. Titrate also a blank on 2 cc. reagent. The calculation of the result is made taking account of the fact that the 2 cc. blood filtrate represent two-thirds of 0.2 cc. or 0.133 cc. blood."<sup>2</sup>

In Table III are given the blood sugar values in mg. per 100 cc. of blood, corresponding to titration values.

In our experience this micro procedure furnishes very satisfactory results, in fact for all practical purposes as accurate as when greater amounts of blood are used. Of course, great care has to be applied to the handling of volumetric utensils, as the accuracy of the method largely depends on the exactness of measurements.

#### IV.

A few remarks concerning *technical details* may be added in conclusion.

<sup>2</sup> This technique was developed by Dr. P. A. Shaffer and is quoted from his Notes on biological chemistry, Washington University School of Medicine, 1926 (a mimeographed laboratory guide for the use of medical students).

*Thiosulfate Solutions.*—A 0.005 N thiosulfate solution cannot be kept unchanged for more than a few days. We prefer to keep a stock of carefully standardized 0.1 N solution and prepare 1:20 dilutions every day or two. When making the dilution the normality factor may be taken into consideration so as to obtain exactly 0.005 N solutions. For example, to make up 500 cc. of 0.005 N solution out of a 0.10045 N stock solution, pipette accurately 25 cc. of stock solution into a 500-cc. volumetric flask, fill up to mark with water, then from a graduated pipette add 2.3 cc. more water.

*Cooling.*—Any agitation of the test-tubes, from the beginning of heating in the water bath up to the addition of acid before titration,

TABLE III.

*Amounts of Glucose Corresponding to Titration Values when 2 Cc. of 1:15 Blood Filtrate and 2 Cc. of Copper Reagent (Modified) Are Heated in Water Bath for 15 Minutes.*

Cc. of 0.005 N thiosulfate.	Tenths of 1 cc. of 0.005 N sodium thiosulfate.									
	0	1	2	3	4	5	6	7	8	9
	Mg. of glucose in 100 cc. of blood.									
0			42	53	63	74	83	91	100	108
1	117	125	134	142	150	159	168	176	185	193
2	202	210	219	227	236	245	253	262	270	279
3	288	296	305	313	322	330	339	347	355	364
4	373	381	390	399	407	416	424	433	441	450
5	458									

should be avoided to minimize reoxidation of cuprous oxide by air. We use test-tube racks, holding eight to twenty tubes, which maintain the tubes erect and stationary during the heating and subsequent cooling.

*It is undesirable to cool below 30°C.* If the 5 cc. solution for a determination contains 1 mg. or more glucose, cooling too far may cause low results, in consequence of incomplete oxidation of the reduced copper by iodine. This reaction is quite rapid until all but 3 to 5 per cent of the cuprous copper is oxidized, but is completed rather slowly at lower temperatures. If the temperature be kept (or raised) between 30 and 40°C. until the acid is added, the oxidation is complete within 2 to 3 minutes. In the case of

sugar solutions of lower concentration (around the normal level of blood sugar) this precaution is of less importance, as the considerable excess of iodine present presses the oxidation quickly to completion even at lower temperatures.

*Effect of Salts.*—It was an early observation of the authors of the Shaffer-Hartmann method that reasonably large amounts of salts in the reaction mixture increase the reduction values, probably due to a decrease of dissolved air, thus diminishing the extent of reoxidation of reduced copper. In many cases one has to deal with sugar solutions containing more or less salts, which at high concentrations may introduce gross errors. For example if 6 per cent sodium sulfate is added to a pure glucose solution, the reduction values are increased 5 to 6 per cent, while the addition of 10 per cent sodium sulfate effects a rise of almost 10 per cent in the reduction. Still higher percentage differences were found at low concentrations (0.040 per cent) of glucose.

Especial attention has been paid to the influence of sodium nitrate. In this laboratory we have used for the precipitation of proteins the Patein-Dufau reagent (6) which represents an acid solution of about 33 per cent mercuric nitrate. On addition of sodium hydroxide, carbonate, or bicarbonate to slightly alkaline reaction, without material change of volume, a solution of about 18 per cent sodium nitrate is produced. For removal of proteins and their cleavage products after direct acid hydrolysis of tissues, as much as half volume of the acid mercuric nitrate solution may be required, thus giving solutions containing about 6 per cent sodium nitrate.

In order to learn the influence of the nitrate, pure glucose solutions were submitted to the entire process of precipitation using varying amounts of mercuric nitrate. The results, given in Table IV, show that plus errors of 4 to 10 per cent may be the consequence of additions of mercuric nitrate precipitant in quantities greater than 1 to 4 volumes of glucose solution. This error can be eliminated either by an adequate dilution of the protein-free filtrate, or—if low concentrations of glucose make a further dilution disadvantageous—a special table of reduction values has to be prepared, using known pure glucose solutions with addition of the amount of salts encountered in the determinations.

*Neutralization of Acid Sugar Solutions.*—Acid solutions—hydrolysates of glycogen for instance—must be neutralized before use for glucose determinations. Without comment it is evident that carbonates and bicarbonates must not be used for neutralization. Phenol red may be used as a convenient indicator for neutraliza-

TABLE IV.  
*Effect of Added Salts on Glucose Determinations.*

Ratio of $\text{Hg}(\text{NO}_3)_2$ solution in the mixture.	Actual amount of glucose.	Glucose found.	Difference due to added salt.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1:3	0.0200	0.0221	+10
1:3	0.0267	0.0282	+5.6
1:3	0.0250	0.0265	+6
1:3	0.0167	0.0176	+5.4
1:3	0.0255	0.0274	+7.6
1:4	0.0200	0.0208	+4
1:4	0.0100	0.098	-2
1:5	0.0200	0.0200	0
1:6	0.0125	0.0125	0
1:6	0.0200	0.0202	+1
1:7	0.0255	0.0255	0
1:10	0.100	0.0102	+2
Added $\text{Na}_2\text{SO}_4$ .			
<i>per cent</i>			
6	0.040	0.045	+12.5
10	0.040	0.047	+17
6	0.080	0.085	+6
6	0.200	0.210	+5
6	0.300	0.320	+6.5
10	0.300	0.328	+9
6	0.400	0.427	+6
10	0.400	0.436	+9

tion with sodium hydroxide as its color in acid solution does not interfere with the sharpness of the end-point at titration. On the contrary it makes the end-point more distinct, and for this reason a few drops may be added before titration by those who have difficulties in observing the disappearance of the color of the iodine-starch in the presence of copper compounds.

## SUMMARY.

1. The great sensitiveness of the reduction values to variations of pH in the system, glucose-alkaline copper solution, is shown.

2. A modification of the Shaffer-Hartmann carbonate-tartrate copper reagent is offered which (1) has a more constant degree of alkalinity, (2) gives higher reduction values, (3) extends the usefulness of the method to lower concentrations of glucose.

3. An adaptation of the Shaffer-Hartmann method for 0.2 cc. of blood is described.

4. Attention is directed to a few details which may cause errors in determinations by these methods.

The author wishes to express his indebtedness to Dr. P. A. Shaffer for his interest and valuable suggestions in the course of this work.

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## THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

### IV. ON THE NATURE OF THE SPECIFIC POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS.

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It was shown in 1917 by Dochez and Avery (1) that there is present in fluid cultures of pneumococci a substance which precipitates specifically in antipneumococcus serum of the homologous type. This "soluble specific substance" was also found in the body fluids of the infected organism, and was even demonstrated in the urine of many patients suffering from pneumonia due to pneumococci of Types I, II, and III. Dochez and Avery also found that the specific substance is not destroyed by boiling, that it is readily soluble in water and precipitable from it by alcohol or acetone, that it does not dialyze through parchment, and that its serological reactions are unaffected by digestion with trypsin.

In later publications from this laboratory (2-4) it has been shown not only that this soluble specific substance appears to belong to the group of polysaccharides, but also that it is possible to isolate from cultures of each antigenic type of *Pneumococcus* a chemically distinct polysaccharide, different in many particulars from those of the other two types, and possessing in the highest degree the property of precipitating the antiserum to the type of *Pneumococcus* from which it was derived. Analogous sugar derivatives with specific properties have been isolated in the case of the Friedländer bacillus (Heidelberg, Goebel, and Avery (5), Mueller, Smith, and Litarczek (6)), the tubercle bacillus (Laidlaw and Dudley (7), Mueller (8)), and even from yeast (Mueller and Tomcsik (9)), and their presence has been shown in *Streptococcus viridans* (Lancefield (10)). The so called "residue

antigens" of Zinsser and Parker (11), first considered as containing in part protein degradation products or nucleic acid derivatives (12), appear also to be specifically reacting polysaccharides.

The immunological significance of this new type of sugar derivative, so frequently encountered among microorganisms, has been discussed elsewhere in papers from this and other laboratories (13-16), and its importance in the study of bacterial specificity demonstrated (17-19). It would accordingly be a matter of great interest to have some knowledge of the chemical structure of the new group of polysaccharides, and the present paper describes a beginning made in the case of the soluble specific substance of Type III pneumococcus.

As previously found (4), this substance may be obtained as a snow-white, amorphous powder, free from nitrogen and ash, and with marked acidic properties. It is precipitated from aqueous solution by strong mineral acids, and is then insoluble in water when dried. An aqueous solution of the sodium salt shows a specific rotation of about  $-34^{\circ}$  and gives precipitates with many heavy metal salts and with barium hydroxide in excess. At a dilution as high as 1 to 6,000,000 it still yields a detectable precipitate with Type III antipneumococcus serum. The specific property disappears when the substance is hydrolyzed by means of acid. The products of hydrolysis are glucose and an acid which was considered to be a condensation product of a hexose and a -uronic acid.

The chief stumbling block to chemical progress with the bacterial polysaccharides has been the small amount of material available for investigation. It has been found possible in the case of the Type III pneumococcus to overcome this objection to some extent. In the first place this microbe produces far more specific substance than do the other two antigenic types of *Pneumococcus*, and in addition its polysaccharide is the easiest of the three to isolate and purify. Furthermore, once the properties of the Type III specific substance had been established, it was considered proper to add glucose to the usual *Pneumococcus* broth (2). In this way a greatly increased growth was obtained, and whereas the yields of specific substance were originally from 6 to 9 gm. per 300 liters of broth, they now rose to 35 to 40 gm. With these increased amounts of material it has been possible to show that the complex

sugar acid mentioned above is not only the chief product of hydrolysis, but that it is apparently the actual unit from which the whole polysaccharide is built up. The structure of the acid has also been partly elucidated.

#### EXPERIMENTAL.

##### *A. Preparation of the Type III Soluble Specific Substance.*

For the preparation of the specific polysaccharide the Type III pneumococcus was grown and the specific substance isolated as in Paper III (4), except that by the addition of 0.3 per cent of glucose to the liquid medium

TABLE I.

Preparation No.	Acid equivalent.	Specific rotation.	Total N.	Reducing sugars on acid hydrolysis.	Ash.	Precipitation with antipneumococcus serum.
33*	343	-30.5°	0.0	73.0	0.0	1:6,000,000
53†	338	-34.0°	0.0	70.0	0.0	1:6,000,000
55†	338	-33.0°	0.0	65.0	0.0	1:6,000,000

\* Preparation 33 was isolated from glucose-free broth.

† Preparations 53 and 55 were obtained from the glucose broth.

far greater growth was obtained. Consequently the yield of purified polysaccharide was greatly improved, 300 liters of broth now furnishing between 35 and 40 gm. The material obtained was identical with that previously described, as will be seen from Table I.

##### *B. Hydrolytic Products of the Type III Soluble Specific Substance.*

1. *Products of Partial Hydrolysis.*—10 gm. of air-dried specific polysaccharide were dissolved in 40 cc. of 75 per cent (by weight) sulfuric acid at 0°. After standing for 21 hours in the ice box the solution was poured into 1 liter of water and the sulfuric acid quantitatively removed with barium hydroxide. The resulting solution was concentrated *in vacuo*. The product was found to be divisible into three distinct fractions. Fraction I was obtained by precipitation from a volume of 50 cc. with a slight excess of barium hydroxide saturated at 60°. Fraction III was also obtained as a barium salt by treating the concentrated, barium-free supernatant liquid from Fraction I at a volume of 20 cc. with 3 volumes of the same barium hydroxide solution. Fraction II represents the supernatant liquid of Fraction III, from which the remaining organic matter was precipitated with basic lead acetate after the excess of barium hydroxide had been removed. The lead was of course removed by means of hydrogen sulfide.

It was found possible further to subdivide Fraction I by precipitating a



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neutralized solution with 10 per cent copper sulfate. From the precipitate (Ia), and the supernatant liquid (Ib), the free acids were obtained by eliminating the copper as its sulfide, the sulfate as barium sulfate, and concentrating to dryness. A summary of the properties of these five fractions will be found in Table II.

None of these fractions reacted specifically with Type III antipneumococcus serum. Since no glucose was found it is evident that the initial hydrolytic products of the Type III specific substance consist of a mixture of sugar acids probably of varying molecular weight, but containing a similar bionic acid unit as shown by the figures for the acid equivalent.

On further hydrolysis with boiling dilute acid these fractions were hydrolyzed to the disaccharide acid (aldobionic acid) described in a later section.

TABLE II.

Fraction No.	Acid equivalent.	$[\alpha]_D$	Reduction (calculated as glucose).
			<i>per cent</i>
I	340*	-12.6°	12.5
Ia	343	-11.0°	11.8
Ib	310	-7.9°	17.2
II	340	+9.2°	20.1
III	340	-8.3°	31.0

\*This value remained unchanged in the presence of excess N/14 NaOH on the water bath.

2. *Products of Prolonged Hydrolysis.*—22.5 gm. of polysaccharide (containing 6.5 per cent of water of hydration) were slowly added with stirring and occasional cooling to 100 cc. of 75 per cent (by weight) sulfuric acid. After complete solution had resulted the mixture was placed in the ice box overnight and was then diluted to 2.7 liters and boiled 5 hours under a reflux. The sulfuric acid was removed quantitatively with barium hydroxide and the barium sulfate washed free from reducing sugars. The combined filtrates were concentrated to 150 cc. *in vacuo* and treated with an excess of basic lead acetate solution. After standing overnight in the cold, the portion precipitated by lead (Fraction I) was filtered off. The filtrate (Fraction II) was treated with hydrogen sulfide, and after the lead sulfide had been washed free from reducing sugars the filtrate was concentrated *in vacuo* and a small remainder of sugar acid (Fraction III) was removed by treating with an excess of basic lead acetate. All three fractions were now freed from lead, and in each case the lead sulfide was washed until free from reducing sugars. After removal of the hydrogen sulfide the

amount of sugar in each fraction was determined by the Shaffer-Hartmann method (20).

Fraction II, which had previously been shown to yield only glucose (4), contained 2.0 gm. of the sugar. Fractions I and III showed 8.7 and 0.2 gm., respectively, calculated as glucose. Since the two latter fractions contain a sugar, as will be shown later, having only one-half the reducing power of glucose their weights are ascertained by doubling the glucose value. On adding the three amounts thus obtained it is seen that 19.8 gm. of reducing sugars are accounted for, or 94 per cent of the theory. The total weight should be  $22.5 \times 0.935 = 21.0$  gm.

*a. Properties of Fraction I.*—A portion of Fraction I was evaporated repeatedly with water *in vacuo* to remove acetic acid and was then reprecipitated as its lead salt. This was freed from lead and evaporated to complete dryness, yielding a friable, amorphous product. This material, which forms by far the major portion of the hydrolytic products of the specific polysaccharide, has been described in a preceding paper (4).

The acid equivalent of the crude disaccharide was determined by titration. 36.2 mg. neutralized 5.04 cc. of  $N/50$  NaOH. This gives an acid equivalent of 361; calculated for  $C_{11}H_{19}O_{10}COOH$ , 356. A micro sugar determination by the method of Shaffer and Hartmann (20) on a sample of 1.92 mg. gave a back titration of 12.68 cc. of  $N/200$   $Na_2S_2O_3$ . This corresponds to copper and glucose equivalents of 2.02 and 0.95 mg., respectively. Reduction 49.5 per cent, calculated as glucose.

0.2882 gm. made up to 15 cc. with  $H_2O$ ,  $l = 2$ ,  $\alpha$ ,  $+0.30^\circ$ .  $[\alpha]_D = +7.8^\circ$ .

*b. Formation of a Morphine Salt.*—0.7 gm. of the dry sugar obtained as above was dissolved in 8 cc. of water and morphine was added in excess. Since the salt could not be made to crystallize from solutions containing water it was evaporated to complete dryness over phosphorus pentoxide and taken up in a mixture of equal parts of absolute methyl and ethyl alcohols. A small amount of dark insoluble gum was filtered off. During prolonged standing in the ice box a crystalline product gradually separated. After recrystallization from the same solvent the salt melted at  $153\text{--}156^\circ$  and was difficultly soluble in the usual anhydrous solvents.

0.2154 gm. substance: 3.7 cc.  $N_2$  at  $23^\circ$ , 760 mm.

0.1003 " " : 0.1968 gm.  $CO_2$  and 0.0557 gm.  $H_2O$ .

Calculated for  $C_{21}H_{39}O_{15}N$  ( $C_{12}H_{20}O_{12} \cdot C_{17}H_{19}O_5N$ ). C 54.26 per cent, H 6.13 per cent, N 2.18 per cent.

Found. C 53.52 per cent, H 6.22 per cent, N 2.03 per cent.

0.3888 gm. diluted to 15 cc. with water gave, in a 2 dm. tube, a rotation of  $-2.48^\circ$ , changing after 24 hours to  $-2.80^\circ$ , where it remained constant.

$[\alpha]_D = -47.9^\circ$ , changing to  $-54.0^\circ$ .

*c. Properties of the Sugar Acid Recovered from the Morphine Salt.*—The morphine salt as prepared above was decomposed by the addition of a slight excess of aqueous ammonia. The resulting crystalline morphine was filtered off and the solution containing the sugar acid was twice precipitated with basic lead acetate. The acid was obtained by evaporating the lead-free product to complete dryness. It formed a snow-white, amorphous residue.

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0.1053 gm. diluted to 15 cc. with water gave a rotation of  $+0.14^\circ$  in a 2 dm. tube.  $[\alpha]_D = +10.0^\circ$ .

2 mg. of substance, analyzed by the micro method of Shaffer and Hartmann (20), gave a titration of 12.90 cc. of  $N/200 \text{ Na}_2\text{S}_2\text{O}_3$  (factor 0.9660, blank 19.90 cc.). Copper equivalent, 2.12 mg., glucose 0.99 mg.; reducing sugar calculated as glucose, 49.5 per cent.

0.0956 gm. substance: 0.1398 gm.  $\text{CO}_2$  and 0.0494 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_{12}\text{H}_{18}\text{O}_{12}$ . C 40.45 per cent, H 5.66 per cent.

Found.                      " 39.85 "      " 5.77 "      "

35.17 mg. of sugar neutralized 4.85 cc. of  $N/50 \text{ NaOH}$ . Acid equivalent, calculated for  $\text{C}_{11}\text{H}_{17}\text{O}_{10}\text{COOH}$ , 356; found, 363.

Except for a possible slight difference in optical rotation the crude disaccharide acid corresponds very closely in its properties with the purified acid.

0.2640 gm. of substance, heated with 12 per cent hydrochloric acid and analyzed by a modification of Pervier and Gortner's method (21) required 5.75 cc. of 0.1  $N \text{ KBrO}_3$ , corresponding to 0.0276 gm. of furfural, or 10.5 per cent. Since glucuronic acid yields about one-third of the amount of furfural liberated by pentoses under corresponding treatment (22), this figure would correspond roughly to the presence in the molecule of about 50 per cent of an acid of the glucuronic type.

The substance has marked acidic properties. It has one-half the reducing power of glucose and gives a strong naphthoresorcinol test. The acid is appreciably soluble in hot ethyl and methyl alcohols, and in hot glacial acetic acid, but fails to dissolve in the other usual organic solvents. On further prolonged hydrolysis it yields glucose. It thus appears to be built up from glucose and a -uronic acid in such a way that one reducing group remains free, and hence differs from any non-nitrogenous, naturally occurring disaccharide derivative hitherto described. Of all known sugar derivatives, it would seem most closely related to desaminochondrosin, the nitrogen-free anhydro derivative of chondrosin, a component of chondroitin sulfuric acid, which, in turn, occurs in mucoproteins (23).

*d. Oxidation of the Sugar by the Method of Willstätter and Schudel (24).—*0.1450 gm. of the sugar acid (recovered from its morphine salt), when analyzed by the method of Willstätter and Schudel, reduced 8.14 cc. of  $N/10$  iodine. Theoretically 1 cc. of  $N/10$  iodine should oxidize 0.0178 gm. of the disaccharide acid. 8.14 cc. therefore correspond to 0.1448 gm. of disaccharide acid. The sugar is obviously quantitatively oxidized and its reducing group must be aldehydic in nature since this method is specific for aldoses. The substance may, therefore, be termed an aldobionic acid.

*e. Oxidation of the Aldobionic Acid with Strong Nitric Acid.*—1.5 gm. of specific polysaccharide were dissolved in 8 cc. of 75 per cent sulfuric acid at 0°. After standing overnight in the ice box the solution was diluted with water until the acid was of normal concentration and was boiled 5 hours under a reflux. The aldobionic acid was isolated over the lead salt as described above. At a volume of 4 cc. its aqueous solution was treated with 10 cc. of 1:1 nitric acid, allowed to stand 15 hours, boiled for 3 minutes, and then quickly evaporated on a large watch-glass over a boiling water bath. No formation of mucic acid could be observed, so the solution of the oxidation product, at a volume of 6 cc., was made strongly alkaline with 40 per cent potassium hydroxide and then acidified with an excess of glacial acetic acid. It was then seeded with a small crystal of potassium acid saccharate and allowed to stand at 0° for 24 hours. The crystals which had formed were filtered off, washed with a few drops of ice water, and dried. 0.20 gm. was obtained. The salt was recrystallized from 1 cc. of boiling water, 0.100 gm. of pure potassium acid saccharate being recovered.

0.0496 gm. dry substance: 0.0173 gm.  $K_2SO_4$ .

Calculated for  $HOOC(CHOH)_4COOK$ . K 15.75 per cent.

Found. K 15.65 per cent.

*f. Hydrolysis of the Disaccharide with Acid.*—1.0 gm. of the dry aldobionic acid was dissolved in 50 cc. of normal sulfuric acid and boiled 20 hours under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide and the filtrate concentrated *in vacuo* and boiled with norit. The clear colorless solution, at a volume of 30 cc., was treated with an excess of basic lead acetate to remove the unaltered aldobionic acid. The filtrate was treated with hydrogen sulfide, filtered, and concentrated to dryness *in vacuo*. The residue was diluted to 20 cc. An analysis by the Shaffer-Hartmann method showed it to contain 0.1980 gm. of reducing sugars calculated as glucose. In a 2 dm. tube the solution gave a rotation of  $+1.07^\circ$ .  $[\alpha]_D = +54.1^\circ$ . The remaining solution was treated with 3.5 mols of phenylhydrazine acetate and heated for 1 hour on the water bath. The entirely crystalline osazone which was formed was filtered off and washed with a few drops of methyl alcohol. The yield was 0.10 gm. The product melted at 203–204°. The initial specific rotation was  $-54.5^\circ$ , mutarotating to  $-30^\circ$  after 48 hours.

From the melting point of the osazone, its direction of mutarotation, and finally from the specific rotation of the sugar solution itself, it is justifiable to conclude that this product of the hydrolysis of the aldobionic acid is glucose, and that the hexose half of the molecule is, therefore, glucose. The other half of the molecule (the sugar acid) is largely destroyed by acid hydrolysis (*cf.* (22)). Whether the saccharic acid identified in the preceding section arises from oxidation of the glucose or of the -uronic acid, or both, cannot be stated as yet.

*g. Oxidation of the Aldobionic Acid with Bromine.*—2.4 gm. of the acid were dissolved in 50 cc. of water and to the solution were added 8 gm. of barium carbonate and 1 cc. of bromine. After 2 days 0.5 cc. more bromine was added. 4 days later the solution was filtered, the excess of bromine blown out with air, and the barium and hydrobromic acid removed quantitatively with sulfuric acid and silver sulfate. The resulting solution, freed from silver and sulfuric acid, showed the presence of 10 per cent of unaltered disaccharide by its reduction value. Thus 90 per cent of the aldobionic acid had been oxidized to the corresponding dibasic acid; *i.e.*, the free reducing group had been oxidized.

It was thought possible to hydrolyze the dibasic acid, but boiling with normal sulfuric acid showed only a slight increase in reducing sugars. It may be, of course, that hydrolysis actually took place with the subsequent decomposition of the aldehydic sugar acid. It has as yet been impossible to isolate in a state of purity either the oxidation product itself, or the products of its hydrolysis. That the -uronic acid portion of the molecule is still intact, however, is indicated by the fact that the oxidation product gives a strong naphthoresorcinol test.

#### DISCUSSION.

In the original communication on the soluble specific substance of Type III pneumococcus (3) it was pointed out that on hydrolysis a product with some of the properties of glucuronic acid was obtained. In a later paper (4) it was shown that glucose was one of the products of hydrolysis, while the other isolated corresponded not with glucuronic acid itself but with a more complex derivative of the glucuronic type, possibly consisting of glucuronic acid combined with a hexose.

The following is now presented as evidence, when considered collectively, that this portion of the hydrolytic products of the Type III specific substance, precipitable by basic lead acetate, is actually a compound of glucose and a hexose-uronic acid.

1. The reducing power of both the crude and the purified anhydrous substance is 50 per cent of that of glucose.

2. The acid equivalent is found to be 363, while the value calculated for  $C_{11}H_{19}O_{10}\cdot COOH$  is 356.

3. As an acid, the substance forms a morphine salt which can be crystallized and purified by recrystallization. The analysis of the salt gives values for carbon, hydrogen, and nitrogen checking closely with the theoretical. The purified acid, recovered from the salt, is scarcely different from the crude material.

4. On prolonged hydrolysis only a small amount of glucose, in addition to unhydrolyzed material, can be isolated, the acid half of the portion hydrolyzed apparently decomposing similarly to glucuronic acid.

5. The reducing group of the disaccharide acid is aldehydic, as shown by the fact that it may be quantitatively determined by the Willstätter-Schudel method. The substance also gives the color reaction with naphthoresorcinol characteristic of the glucuronic acid type.

Since the easily isolable mucic acid is not formed on hydrolysis and oxidation with nitric acid, the acid portion of the molecule can scarcely be galacturonic acid. The saccharic acid actually recovered from the oxidation mixture certainly arises at least in part from the glucose fraction of the molecule; whether the acid portion takes part in its formation or gives rise to some other soluble acid must be left for future work to determine.

As to the position of the union of the glucose to the sugar acid, the evidence at hand does not permit any conclusion. The linkage may be either through the reducing group of the glucose, or through the reducing group of the sugar acid. That the union is glucosidic is indicated by the fact that on further hydrolysis the reducing power of the aldobionic acid increases to about 65 per cent before dropping owing to destruction of the hexose-uronic acid liberated.

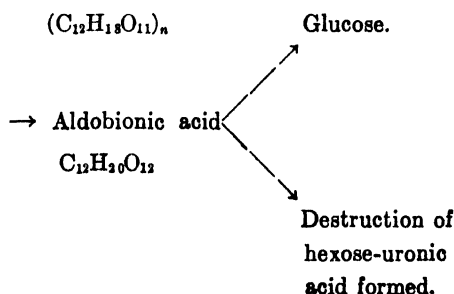
If an analysis be made of the quantitative data obtained on the hydrolysis of the original polysaccharide (p. 617), it becomes evident that the aldobionic acid accounts for about 85 per cent of the total products of hydrolysis, while only 9.5 per cent is glucose, and 5.5 per cent unaccounted for. Thus by far the major portion of the polysaccharide seems to be constructed from molecules of this disaccharide acid. Now it has also been found (p. 619) that the aldobionic acid itself slowly hydrolyzes, to the extent of about 1 per cent per hour, on boiling with dilute mineral acid. It is therefore not illogical to assume that the 9.5 per cent of glucose liberated during the hydrolysis of the polysaccharide owes its origin, not to a separate part of the carbohydrate molecule, but chiefly to a secondary reaction involving the aldobionic acid. This assumption is all the more justified by the fact that no glucose is split off during the preliminary hydrolysis by 75 per cent

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sulfuric acid in the cold. Since, also, these partial hydrolysis products show, by their acid equivalents, one carboxyl group for every two sugar nuclei, it would seem that the polysaccharide as a whole is built up of units of the aldobionic acid.

The condensed or polysaccharide form of a hexose-hexose-uronic acid should have the formula  $(C_{12}H_{20}O_{12})_n - (n - 1)H_2O$ , or  $(C_{12}H_{18}O_{11})_n$ . A substance of this composition should have an acid equivalent of 338 and a carbon and hydrogen content of 42.6 per cent and 5.4 per cent respectively. These figures are practically identical with actual analytical values obtained ((4) p. 733, Preparation 33 II. Acid equivalent, 340; C, 42.7 per cent; H, 5.3 per cent). Thus one may justifiably conceive of the Type III soluble specific substance as a condensation product of the disaccharide acid,  $C_{12}H_{20}O_{12}$ , built up in such a way that the carboxyl groups remain free. Hydrolysis by means of acid follows the course:

Polysaccharide acid  $\rightarrow$  Intermediate acids



In view of the evidence collected it is believed that the specific polysaccharide of the Type III pneumococcus is a definite chemical individual composed of units of a difficultly hydrolyzable aldobionic acid in which glucose and a hexose-uronic acid are combined in such a way that one aldehydic group and the carboxyl remain free. The polysaccharide is thus unusual not only in its possession of immunological specificity, but in its chemical constitution as well.

The question as to whether its unusual structure bears any relation to its immunological properties must be left for future work on this and other specific polysaccharides for a decision.

## SUMMARY.

1. The soluble specific substance of Type III pneumococcus is shown to yield on hydrolysis a small amount of glucose and chiefly a disaccharide acid of a type not hitherto observed in any non-nitrogenous polysaccharide.

2. The disaccharide acid corresponds to the formula  $C_{12}H_{20}O_{12}$  and contains one carboxyl group and one aldehydic reducing group in the molecule. It yields a crystalline morphine salt and appears to consist of 1 molecule of glucose condensed with 1 molecule of a hexose-uronic acid through one of the two reducing groups.

3. The specific polysaccharide is believed to be built up of units of this aldobionic acid, and thus to belong to a new type.

In conclusion the writers wish to express their gratitude to Dr. P. A. Levene for his many helpful suggestions, and to Dr. W. A. Jacobs for his assistance as well.

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# ON LIVER AMYLASE AND ITS PROBABLE RÔLE IN THE REGULATION OF BLOOD SUGAR.

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The question of the effect of insulin and adrenalin on the activity of extracted liver amylase is fundamentally important, for if it could be shown that a consistently demonstrable effect exists, our conception of the mechanism which regulates the level of blood sugar in the animal body would be greatly simplified.

In 1921, Langfeldt (1) reviewed the subject of adrenalin hyperglycemia and attempted to explain its mechanism (in part at least) as a direct hormonal action of adrenalin on the amylase in the liver. The evidence presented was a series of experiments performed *in vitro* with an amylase preparation obtained from calf livers. The activity of this preparation at hydrogen ion concentrations from pH 5.8 to 8.0 was studied with and without the addition of chloride, and with the addition of adrenalin, thyroiodine, and pituitary extract. The results obtained indicated that the addition of adrenalin not only accelerated the action of the amylase but caused the maximum amount of hydrolysis to occur at pH 7.7, whereas the optimum pH was 6.2 with phosphate alone and 6.8 with phosphate and chloride. A concentration of adrenalin as low as 1 to 5,000,000 apparently caused considerable increase in hydrolysis and shift in pH optimum provided thyroiodine was added. Pituitary extract was without effect. In his interpretation of these experiments, considerable emphasis was placed on the shifting of the optimum pH from a point without the range of normal body fluids to one within this range, and it was considered likely that adrenalin acted thus directly on the liver amylase in the body.

A confirmation of Langfeldt's work has been reported by Cam-

midge and Howard (2), and additional data are given by them to show that insulin inhibits the rate of hydrolysis of starch *in vitro* by liver amylase. They compared the action of liver amylase on glycogen and starch and found no essential difference in behavior of the two substrates, hence used starch because it was easily obtainable. They also attacked the problem of blood sugar regulation from another angle by making a study of the effect of Na, K, Ca, and Mg chlorides on the amount of starch hydrolyzed by liver amylase in phosphate buffer mixtures of pH 6.2 to 8.0. A rather unique result was obtained when a mixture of these chlorides (simulating the concentration of the chlorides of blood) was added. A minimum amount of hydrolysis occurred at pH 7.35 with maxima at 6.5 and 8.0, whereas a maximum occurred at 6.8 with minima at 6.5 and 8.0 when NaCl alone was used.

More recently (1926) Visscher (3) has added some data by repeating a part of Langfeldt's work using an amylase preparation from rabbit liver. His experiments were better controlled than those of the earlier workers in that simultaneous hydrolyzing mixtures unmodified by the addition of adrenalin were run as checks on those modified by the addition of adrenalin. He also accounted for the reducing substances added to the working mixture by the enzyme itself by determining the copper-reducing value of the mixture at the start of the experiment. A further check on the effect of the buffer on the reducing action of glucose on the copper reagent (Shaffer-Hartmann) was made and correction for it made in reckoning the final results. The data indicate that adrenalin increases the amount of substrate (glycogen) hydrolyzed, but that it causes no shift in the optimum hydrogen ion concentration for maximum activity.

Rockwood (4) has reported the acceleration of the action of salivary amylase by adrenalin, but no experimental data are given.

In spite of all the evidence that adrenalin increases the hydrolytic action of liver amylase, and insulin inhibits it, I am compelled, from the experimental evidence reported below to present the opposite view; namely, that neither adrenalin nor insulin has any effect in concentrations which are at all comparable to physiological concentrations. In 1925, I began a series of experiments designed to test the results obtained by Langfeldt and Cammidge and Howard. In order to become familiar with

the technique before working with liver amylase, commercial pancreatin was used for the enzyme with glycogen as the substrate. The buffer consisted of  $\text{Na}_2\text{HPO}_4$ , 0.04 M and citric acid 0.006 M (final concentrations), pH approximately 7.0. Three 50 cc. volumetric flasks were made up as follows:

- Flask 1. Buffer, adrenalin 1 to 125,000, and glycogen 0.17 per cent.  
" 2. " no adrenalin and glycogen 0.17 per cent.  
" 3. " adrenalin, but no glycogen.

Pancreatic enzyme (Fairchild Brothers and Foster) to make a final concentration of 0.005 per cent aqueous extract of the powder was added to all three, the flasks made up to volume, and incubated at  $37.5^\circ\text{C}$ . Samples were taken at intervals, and sugar was determined by the Shaffer-Hartmann (5) micro method. When the results were reckoned, as recommended by Davidson (6), by amount of hydrolysis per unit time, the flask containing the adrenalin showed a rate approximately twice that of the control (No. 2). Flask 3 served to show the amount of reduction of the copper reagent caused by the enzyme and adrenalin without glycogen and calculations were made with this deducted.

When the same type of experiment was repeated with physiological saline solution diluted in the same manner and substituted for the adrenalin (since commercial adrenalin is dissolved in this medium) the same acceleration of hydrolysis was observed as in the experiments in which adrenalin was used. (See Fig. 1.) When, also, the experiment was conducted in a medium containing NaCl in approximately 0.1 M solution the accelerating effect of the adrenalin disappeared.

The effect of insulin was investigated in a similar manner and found to cause acceleration when no chloride was added, but the effect was no longer observable when 0.1 M NaCl was added to both the flask containing the insulin and the control. It seemed obvious that, so far as pancreatic amylase was concerned, the modification in its activity on addition of insulin or adrenalin was due to the chloride and not to the hormones themselves. (See work of Sherman and coworkers (7) for the effect of chloride on pancreatic amylase.)

It remained then to test the effect of both insulin and adrenalin on liver amylase, assuming that the amylase obtained from liver

possessed a susceptibility to their influence not possessed by pancreatic amylase. In order to study liver amylase it was first necessary to form some conception of the activity of the amylolytic ferment of blood, since it is practically impossible to free liver entirely from blood which becomes trapped in the capillaries. Thorough perfusion with distilled water has been fairly effective, but one can never feel sure that all the blood is removed. A series of experiments showed that the amylase preparation made

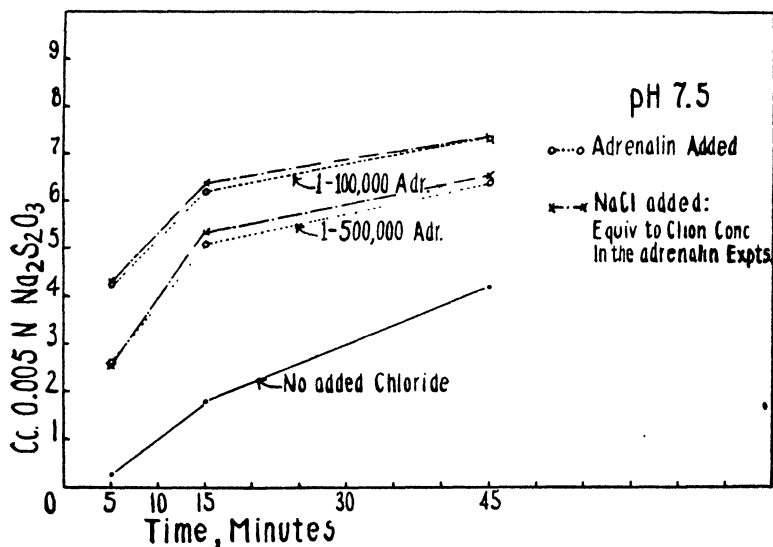


FIG. 1. A comparison of the effect of NaCl and adrenalin on pancreatic amylase and glycogen showing that the accelerating effect of the adrenalin was directly proportional to its chloride content.

from a liver which had not been perfused was capable of hydrolyzing approximately 20 per cent of a glycogen substrate in 2 hours, while the preparation from a perfused liver required 18 hours to produce a comparable amount of hydrolysis. Blood serum was still more active, but since it had not been put through an extraction process the conditions were not comparable. However, it may be said that blood serum (unmodified) is at least fifty times as active as the best liver preparation from well perfused liver that I have been able to obtain, comparable concentrations being calculated on the basis of the weight of liver taken and volume of serum.

Incidentally, the effect of adrenalin on the amylolytic activity of serum was tested, and also on the postmortem production of reducing substances by liver hash with and without added glycogen. Entirely negative results were obtained in both experiments.

With these preliminary tests in mind a more thorough series of experiments was performed. Starch has been used as the substrate, since it was found that it was more readily attacked by animal amylases than glycogen, and it was desirable to take advantage of any means of aiding the very feeble amylolytic power of preparations made from well perfused livers. Two experiments not described were performed with glycogen as the substrate, but the amount of hydrolysis was so small that their value is questionable. However, so far as being affected by the addition of adrenalin, they were entirely negative.

The enzyme preparations used were dialyzed to free them from reducing substances. This dialysis was carried on in a medium of 50 per cent alcohol, since it has been shown by Sherman and Schlesinger (8) that pancreatic amylase is stable in this medium. No deterioration of salivary and liver amylases in 50 per cent alcohol was observed in the experiments described here, while they deteriorated rapidly in water.

The citrate buffer system used in the earlier experiments was discontinued because the citrate affected the reducing ability of glucose on the copper reagent. Phosphate buffers were used instead since in the concentration employed (0.04 M) they did not appreciably affect the determination of sugar.

It is regretted that the results have been negative with respect to any characteristic modification of the enzyme's activity by insulin, adrenalin, or the four salt mixture.

#### EXPERIMENTAL.

A series of eight 50 cc. volumetric flasks was made up with all the ingredients except the insulin and adrenalin as shown on page 630.

The flasks were made up to volume with distilled water and well mixed. Two portions of 15 cc. each were taken from each flask and put into clean, dry, 25 × 200 mm. test-tubes. 1 cc. of diluted insulin solution (Lilly) was added to one series of eight tubes, and 1 cc. of diluted adrenalin (Parke, Davis and Company) added to the other series. The eight flasks with the remaining

20 cc. each received 1.3 cc. of distilled water to equalize the dilution. They served as controls on the other two series. After incubation, sugar determinations were made by the Shaffer-Hartmann (5) micro method. The time and temperature of incubation are stated for each experiment separately.

Flask No.	Buffer.	pH	In each flask except No. 8.
1	10 cc. of $\text{Na}_2\text{HPO}_4$ -	5.8	5 cc. 2 per cent soluble
2	$\text{KH}_2\text{PO}_4$ mixtures to	6.2	starch. 5 cc. 1 M NaCl,
3	give the required pH.	6.6	or 5 cc. Cammidge salt
4		7.0	mixture. 4 drops tol-
5		7.4	uene. Enzyme 1 to 3
6		7.8	cc. (Quantity stated
7		8.2	for each experiment.)
8		7.0	Control on enzyme, chlo-
			ride but no starch added.

Two enzyme preparations were used. Both were made from fresh dog liver which had been thoroughly perfused with distilled water.

Preparation A was made by freezing the tissue (after perfusion) and grinding to a pulp in a mortar, extracting with an equal volume of 60 per cent ethyl alcohol for several days in the refrigerator, centrifugalizing, and dialyzing the supernatant fluid. The dialysis was made in collodion sacs against 50 per cent alcohol for 6 days with one change per day.

Preparation B was made by freezing and grinding, and then drying the pulp in a current of air at room temperature. The material so obtained from a 360 gm. liver was extracted with two portions of toluene of 150 cc. each, and then with two portions of absolute alcohol of 100 cc. each. It was allowed to dry, ground fine in a mortar, and extracted with 50 per cent alcohol for 4 days at room temperature. The pH was adjusted to 6.0 by adding a small amount of 0.1 N NaOH. (This method is essentially that of Wiechowski (9) except that alcohol instead of glycerol was used for the extraction.) The alcoholic extract was dialyzed in the same manner as Preparation A.

The concentration of protein in both of these preparations was sufficiently low to allow a direct determination of the sugar formed by the action of the enzyme on the buffered starch solutions.

5 cc. of the working mixture and 5 cc. of copper reagent gave determinations ideally within range of the micro method.

The starch solution used was made from soluble starch prepared from Baker's arrowroot starch according to the method of Small (10). The same solution of approximately 2 per cent strength (0.2 per cent in the working mixture) was used in all experiments.

Since the object of the experiments was a comparative study of the activity of the enzyme under different conditions, the results obtained have been expressed in number of cc. of 0.005 N  $\text{Na}_2\text{S}_2\text{O}_3$ , representing the amount of copper reduced, and consequently the sugar formed.

To determine the effect of the buffer and added chloride on the determination of sugar, a series containing boiled enzyme and added sugar (0.2 mg. per cc. of working mixture) was incubated

TABLE I.  
*Titration Differences in Cc. of 0.005 N  $\text{Na}_2\text{S}_2\text{O}_3$ .*

Flask No.	pH	NaCl	Four salt mixture.	No added chloride.
1	5.8	3.70	4.00	2.70
2	6.2	5.40	5.70	3.15
3	6.6	7.05	7.35	2.70
4	7.0	7.40	7.80	2.05
5	7.4	7.50	7.80	1.25
6	7.8	6.85	7.30	0.75
7	8.2	5.35	6.20	0.40
8	7.0	Blank containing no starch.		

for 21 hours. The sugar was recovered quantitatively, and there was no variation caused by the buffer mixtures (0.04 M) at the different pH.<sup>1</sup>

*Experiment 1.*—A comparison was made of the effect of 0.1 M NaCl, and the four salt mixture described by Cammidge, on the activity of liver amylase Preparation B. (The solution of the salt mixture contained 6 per cent NaCl, 0.42 per cent KCl, 0.24 per cent  $\text{CaCl}_2$ , and 0.18 per cent  $\text{MgCl}_2$ .)

<sup>1</sup> Visscher (3) has reported that potassium dihydrogen phosphate interfered with the reduction of copper by glucose when the Shaffer-Hartmann macro method for determining glucose was used. This interference is not present when the micro method using the combined tartaric acid reagent is employed and the buffer mixtures are as dilute as 0.04 M.



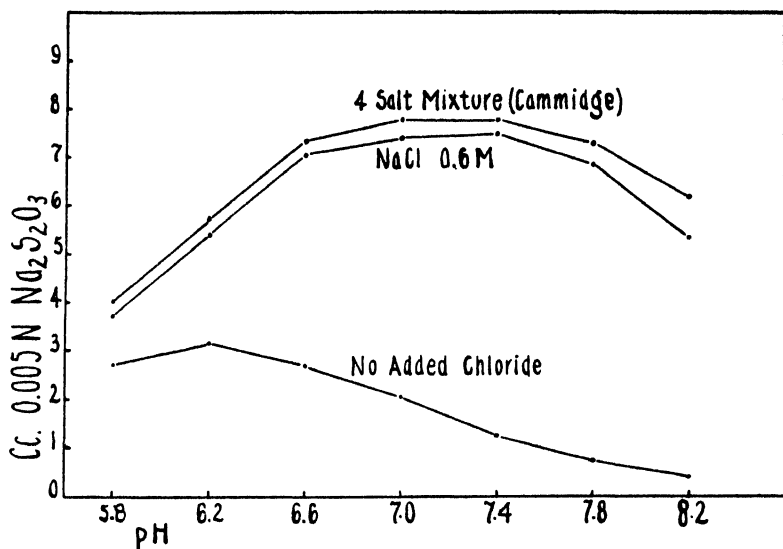


FIG. 2. A comparison of the effect of 0.6 M NaCl and the four salt mixture, described by Cammidge, on liver amylase Preparation B and starch. The optimum pH is the same for both.

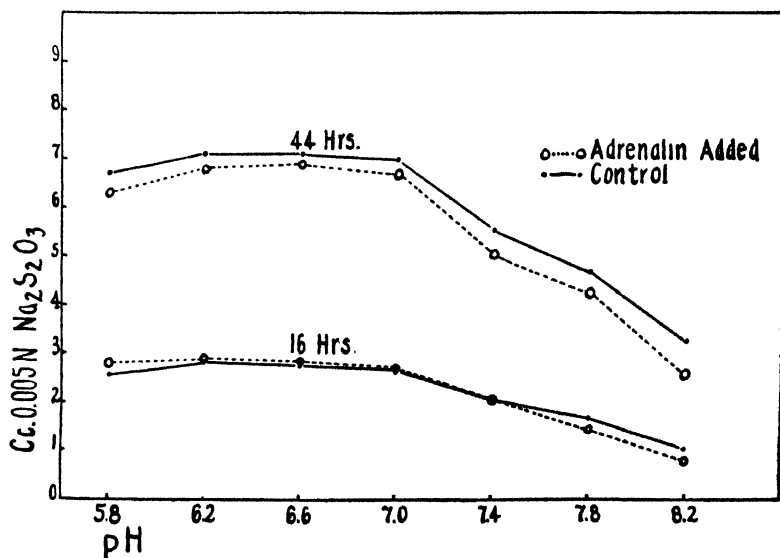


FIG. 3. The absence of any significant effect of adrenalin on the activity of liver amylase Preparation A *in vitro* in the presence of 0.1 M NaCl.

A set of eight flasks was set up as described above except that the chloride was omitted. After making up to volume, two 15 cc. samples were taken out of each flask and put into large test-tubes. 1.5 cc. of 1M NaCl were added to each tube of one set, and 1.5 cc. of the four salt mixture to the other. 2 cc. of distilled water were added to each flask. The whole was incubated 20 hours at 37°C., and the sugar formed was then determined.

TABLE II.

Flask No.	pH	Adrenalin added.		No adrenalin added.	
		16 hrs.	44 hrs.	16 hrs.	44 hrs.
1	5.8	2.80	6.30	2.55	6.70
2	6.2	2.90	6.80	2.70	7.10
3	6.6	2.85	6.90	2.80	7.10
4	7.0	2.70	6.70	2.65	7.00
5	7.4	2.05	5.05	2.05	5.55
6	7.8	1.45	4.25	1.70	4.70
7	8.2	0.80	2.55	1.05	3.25

TABLE III.

Flask No.	pH	Adrenalin added.		Insulin added.		Control.	
		20 hrs.	44 hrs.	20 hrs.	44 hrs.	20 hrs.	44 hrs.
1	5.8	3.15	6.50	3.05	6.40	3.05	6.40
2	6.2	3.55	7.30	3.30	6.85	3.55	7.05
3	6.6	3.45	7.20	3.35	7.10	3.45	7.00
4	7.0	3.20	7.10	3.10	6.80	3.20	6.70
5	7.4	2.65	5.55	2.60	5.70	2.65	5.50
6	7.8	2.40	4.20	2.30	3.65	2.40	3.85
7	8.2	1.30	2.65	1.30	2.75	1.30	2.75

The optimum activity occurred between pH 7.0 and 7.4 with both chloride mixture and NaCl. When no chloride was added the optimum appeared at 6.2. This is in agreement with Langfeldt's work excepting that there was no peak at 6.2 for the "phosphate diastase" when chloride was added (Fig. 2, Table I).

*Experiment 2.*—Adrenalin was added to the working mixture so that the final concentration was 1 to 100,000. NaCl was added to make a final concentration of 0.1 M, and 3 cc. of enzyme Prepa-

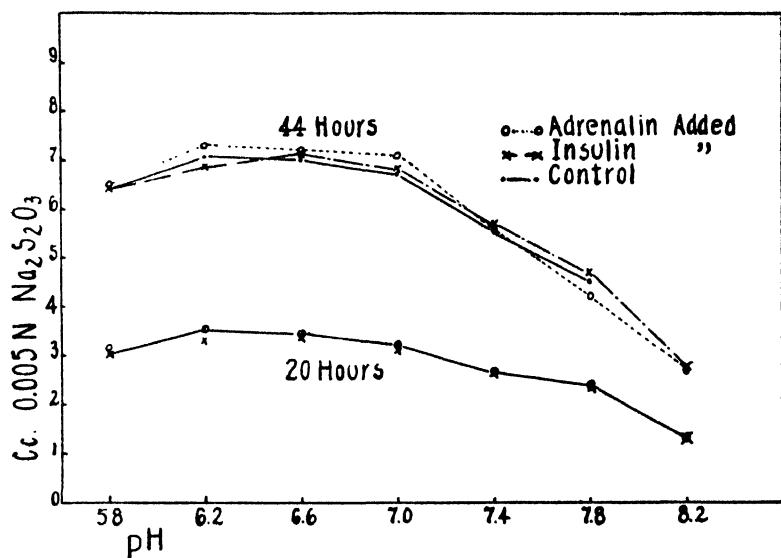


FIG. 4. A comparison of the effect of adrenalin and insulin on liver amylase Preparation A and starch in 0.1 M NaCl.

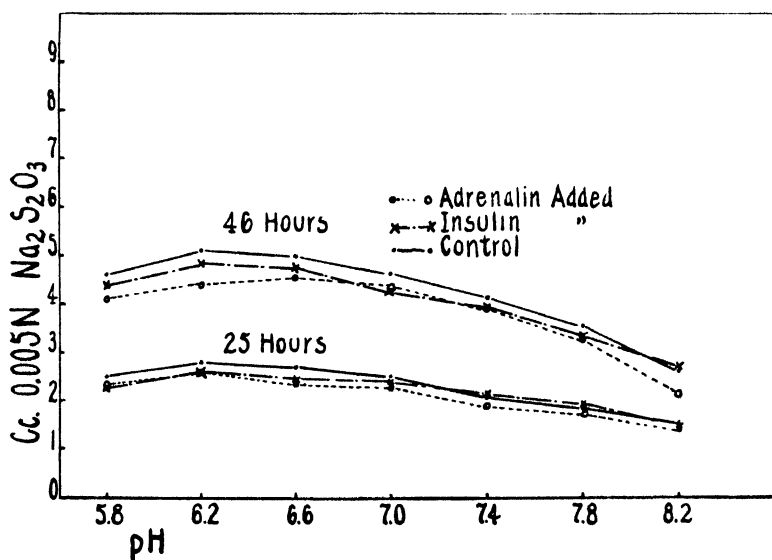


FIG. 5. A comparison of the effect of adrenalin and insulin on liver amylase Preparation A and starch with Cammidge four salt mixture.

ration A to each 50 cc. This was incubated at 36° and samples taken at 16 and 44 hours.

The H ion optimum remained consistently at pH 6.2 to 6.6 for both series, and the amount of hydrolysis was not consistently affected by the addition of adrenalin (Fig. 3, Table II).

*Experiment 3.*—This was a repetition of Experiment 2, except that a third series was included. Insulin ( $\frac{1}{250}$  unit) was added to each 15 cc. of working mixture in it. It was incubated at 37° (Fig. 4, Table III).

TABLE IV.

Flask No.	pH	Adrenalin added.		Insulin added.		Control.	
		25 hrs.	46 hrs.	25 hrs.	46 hrs.	25 hrs.	46 hrs.
1	5.8	2.35	4.10	2.25	4.40	2.50	4.60
2	6.2	2.60	4.40	2.60	4.85	2.80	5.10
3	6.6	2.35	4.55	2.45	4.75	2.70	5.00
4	7.0	2.30	4.35	2.40	4.30	2.50	4.65
5	7.4	1.90	3.90	2.15	3.95	2.10	4.15
6	7.8	1.70	3.25	1.90	3.35	1.85	3.55
7	8.2	1.40	2.10	1.45	2.70	1.50	2.60

TABLE V.

Flask No.	pH	Adrenalin added.	Insulin added.	Control.
1	5.8	4.35	4.75	4.95
2	6.2	6.20	6.85	7.25
3	6.6	8.30	8.45	9.05
4	7.0	9.35	9.55	9.55
5	7.4	9.05	9.50	9.55
6	7.8	8.00	8.65	8.65
7	8.2	6.75	7.40	7.25

*Experiment 4.*—This was the same type of experiment as No. 3 except that the four salt mixture described by Cammidge was used for the activator instead of NaCl, and 2 instead of 3 cc. of enzyme Preparation A were used in each 50 cc. of working mixture. It was incubated at 35°.

There was less hydrolysis in this experiment because less enzyme was used than in Experiment 3. The curves plotted from the data show no significant differences from those of Experiment 3 (Fig. 5, Table IV).

*Experiment 5.*—A repetition of Experiment 4 was performed except that 1 cc. of enzyme Preparation B was used in each 50 cc. of the working mixture. It was incubated 21 hours at 37°.

The optimum occurred at pH 7.0 to 7.4 as it did in Experiment 1, in which the same enzyme (Preparation B) was used (Fig. 6, Table V).

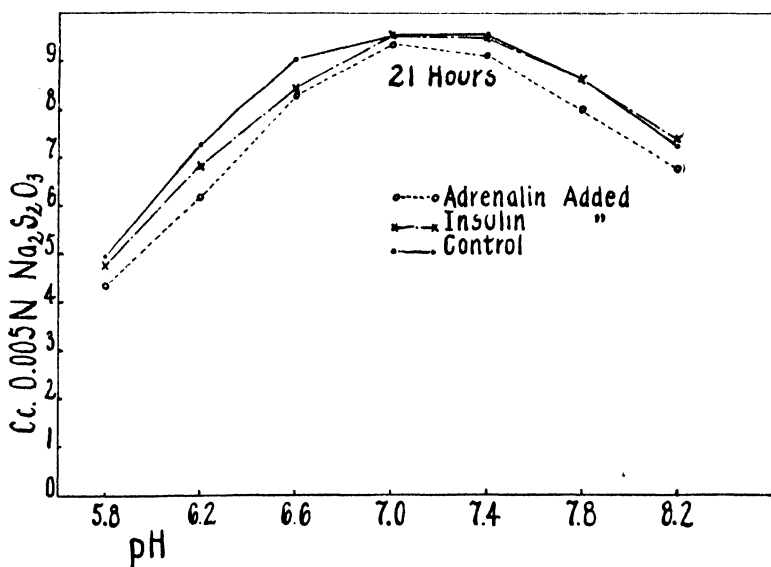


FIG. 6. A comparison of the effect of adrenalin and insulin on liver amylase Preparation B and starch with Cammidge four salt mixture.

#### DISCUSSION OF RESULTS.

It will be seen that most of the experiments in the series modified by adrenalin and by insulin show slightly less hydrolysis than the control. The amount of difference is considered to be too slight to be significant.

In the experiment in which the Cammidge four salt mixture was used the apparent slight increase in hydrolysis is probably due to two factors: greater chloride concentration than in 0.6 M NaCl alone, and a greater amount of copper reduction due to the greater salt concentration itself. There is no inversion of the curve with a minimum at pH 7.4.

One of the most striking features is the feeble amylolytic power

of the liver amylase. It will be noted that Preparation B was more active than Preparation A. The liver from which Preparation B was made retained more blood than the liver from which Preparation A was made, and it is possible that all the amylolytic action observed was due to blood. It is also possible that much of the ferment was lost in the preparation, although this seems unlikely. Since glycogen cannot be synthesized *in vitro* it seems more probable that its hydrolysis in the liver cell is in some way dependent on cell structure, and that the hydrolytic as well as the synthetic ability is destroyed when the cell structure is destroyed.

The results of all experiments tend to show that the mechanism of the regulation of blood sugar is likely to be found elsewhere than in the *in vitro* effects of adrenalin and insulin (as well as chloride mixtures) on liver amylase and conclusions drawn by previous workers regarding such effects have not been confirmed.

#### SUMMARY.

1. The effect of insulin and adrenalin on the activity of extracted liver amylase has been investigated under controlled conditions. No consistent or noteworthy effect was observed.

2. A direct comparison of the activating influence of tenth molar NaCl and the four salt mixture described by Cammidge and Howard has been made. The results show no particular difference between the two except that there was a slightly greater amount of hydrolysis with the four salt at all H ion concentrations than with NaCl.

3. The hydrogen ion optimum for maximum activity was unaffected by any of the added variables (adrenalin, insulin, and different chlorides), but appeared to be a characteristic of the particular enzyme preparation used.

I greatly appreciate the help received from Professor P. A. Shaffer and Dr. T. E. Friedemann during the course of this work.

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# EFFECT OF HYDROGEN ION CONCENTRATION UPON THE RATE OF DESTRUCTION OF VITAMIN B UPON HEATING.\*

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In the work of earlier investigators, summarized elsewhere,<sup>1</sup> it has been shown that alkalinity accelerates the destruction of vitamin B upon heating, but we have seen no record of experiments in which this has been studied quantitatively with reference to the actual or thermodynamic concentration or "activity" of hydrogen or hydroxyl ions.

In a previous series of experiments carried out in this laboratory<sup>2</sup> quantitative investigation had shown a destruction of approximately 20 per cent of the vitamin B present in tomato juice when this was heated for 4 hours at 100° at its natural acidity of pH 4.28; with a steadily increasing rate of destruction at higher temperatures under otherwise constant conditions.

The purpose of the experiments here described was to determine quantitatively the effect upon the rate of destruction of vitamin B when heated at 100° in the same medium but with definite changes of hydrogen ion concentration in the direction of decreasing acidity and increasing alkalinity, the natural reaction of pH 4.28 being shifted successively to pH 5.2, pH 7.9, pH 9.2, and pH 10.9. These changes in the reaction of the medium resulted in successive increases in the rate of destruction of the vitamin.

The methods and results of the measurement of these effects are here presented in very condensed form.

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<sup>1</sup> Sherman, H. C., and Smith, S. L., *The vitamins*, New York, 1922.

<sup>2</sup> Sherman, H. C., and Grose, M. R., *J. Am. Chem. Soc.*, 1923, xlv, 2728.



## EXPERIMENTAL.

*Preparation and Heating of Solutions of Vitamin B.*—The filtered juice of canned tomatoes was selected as constituting a source of vitamin B as suitable and constant as could be found according to our present knowledge, and possessing the added advantage of permitting direct comparison of results with those of other investigations upon the thermostability of the vitamins which have been and are being conducted in this laboratory.

The juice was obtained by pressing through cheese-cloth, then clarified by filtering first through a coarser filter paper and finally through Schleicher and Schull's No. 590. In this way a clear yellow filtrate was obtained. All filtrations were carried out at ice box temperature, about 10°C. Adjustments of hydrogen ion concentration by addition of 0.4 normal sodium hydroxide solution were also performed at this temperature in order to minimize any changes which might tend to result from local excess of the alkali during its careful dropwise addition. All solutions were brought to room temperature (20–23°C.) for electrometric determinations of pH, which were made by means of a Leeds and Northrup potentiometer, using electrode vessels similar to those described by Wilson and Kern.<sup>3</sup> A saturated calomel electrode was employed because of its constancy as shown by Fales and Mudge;<sup>4</sup> and electrodes carefully platinized according to Beans and Hammett.<sup>5</sup>

After careful measurement of its pH, each solution was rapidly heated to the temperature of boiling water and maintained at this temperature for the desired length of time (1 hour or 4 hours), then cooled at the same rate at which it had been heated, and the pH again determined at room temperature. Then to prevent the continued action of the alkali upon the vitamin in the solution while stored for feeding, the added alkali was neutralized by standard acid and the solution stored at ice box temperature until used. As was to be expected, the heating of the tomato juice at its natural acidity did not appreciably change its pH, as determined at the same temperature before and after the heating; but

<sup>3</sup> Wilson, J. A., and Kern, E. J., *Ind. and Eng. Chem.*, 1925, xvii, 74.

<sup>4</sup> Fales, H. A., and Mudge, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2434.

<sup>5</sup> Beans, H. T., and Hammett, L. P., *J. Am. Chem. Soc.*, 1925, xlvii, 1215.

when alkali had been added the heating resulted in the consumption of a very small proportion of the added alkali by reaction with constituents of the filtered tomato juice, and a consequent slight shifting of the pH during the time of the heating. The change was apparently too small to have any significant influence upon the results, and the pH shown in the tabulation of data is, in each case, that measured at the beginning of the heating experiment.

*In order to determine whether oxidation was a factor in the destruction of vitamin B here studied, additional comparisons were made. Part of the tomato juice which had been brought to pH 9.2 was heated in loosely stoppered flasks, while a second part was heated in an atmosphere of nitrogen, using the method adopted by Quinn<sup>6</sup> for the complete removal of oxygen and the maintenance of strictly anaerobic conditions for 2 hours before and during the heating, and carefully keeping all other conditions exactly the same for the two portions of heated juice.*

*Feeding Experiments for Quantitative Measurement of Relative Amounts of Vitamin B after Different Treatments.*—Relative amounts of vitamin B were determined by means of quantitative feeding experiments with young albino rats according to the general method which had been quite critically studied in a previous investigation in this laboratory.<sup>7</sup> The initial age of the experimental animals was always 28 to 29 days, and their initial weights usually 38 to 50 gm. All of the feeding experiments were made in pairs upon litter mates of essentially the same initial size, one being fed material which had been heated at its natural acidity and the other material which had been heated at a different pH. Such comparisons were repeated as often as necessary to find and establish with satisfactory quantitative exactness the relative amounts of the two samples required to furnish the same amount of vitamin B; namely, in this investigation, the amount needed to maintain the body weight of the experimental animal without net gain or loss during an experimental feeding period of 8 weeks duration. That such results could be interpreted in terms of vitamin B alone, was ensured by supplying the experimental animals *ad libitum* with a basal diet which has been shown

<sup>6</sup> Quinn, E. J., Dissertation, Columbia University, New York, 1925.

<sup>7</sup> Sherman, H. C., and Spohn, A. A., *J. Am. Chem. Soc.*, 1923, xlv, 2719.

to be not only adequate but approximately optimal (for rats) as regards all other factors.<sup>7</sup> The composition of this basal diet (Diet 107) was as follows: extracted casein, 18; Osborne and Mendel<sup>8</sup> salt mixture, 4; butter fat, 8; cod liver oil, 2; starch, 68 per cent.

Each animal was kept in a separate cage with raised screen bottoms to prevent access to excreta. The weight of each animal was recorded at least once each week. The intake of the basal diet, as well as of the food furnishing the vitamin B, was also

TABLE I  
*Percentage Destruction of Vitamin upon Heating, as Influenced by the Reaction of the Solution in Which It Is Heated.*

Reaction of solution when heated.	Percentage of vitamin B destroyed.	
	In 1 hr. at 100 °C.	In 4 hrs. at 100 °C.
<i>pH</i>		
4.28	About 9 (?).	20
5.2	" 10 (?).	31
7.9	32	70
9.2	66	*
10.9	90-100	*

\* From the other results it is apparent that in these solutions, more alkaline than pH 9, the destruction of vitamin B would be practically complete in less than 4 hours at 100°C. When only a very small proportion remains, it cannot be determined quantitatively by present methods.

recorded. The tomato juice was carefully measured by means of a graduated pipette into a small feeding cup and placed in the cage at a nearly uniform hour each day, except Sundays. Prompt and complete ingestion of the daily allowance of vitamin B was secured (when necessary) by removing the drinking water until the tomato juice had been consumed.

### *Results.*

The average results, in terms of percentage destruction of vitamin B upon heating at 100° for the time and at the reaction shown, are summarized in Table I. Since for the sake of brevity

<sup>8</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

the averages only are given, it may be added that these represent the data of over 200 individual experiments, each of 8 weeks duration except in those cases in which because of high destruction of vitamin B the animal failed to survive the full period of experimental feeding.

The individual data are as consistent throughout as can be expected at the present stage of quantitative development of the method.

Because of this limitation of method a relatively small (say 10 per cent) or a relatively large (90 to 100 per cent) destruction of the vitamin cannot be estimated as satisfactorily as can differences in the neighborhood of 30 to 70 per cent. Thus the experimental data plainly reveal the difference in the rate of destruction at pH 4.28 and pH 5.2 when the heating at 100° was continued for 4 hours, but not when the solutions were heated for only 1 hour. The failure of the method to reveal the difference which undoubtedly existed at the end of the hour, but escaped experimental measurement, would be misleading if these data stood alone. They also lead to an ambiguous result when the results of heating for 1 hour *vs.* 4 hours are compared since on the face of the data it would appear that the longer heating increased the destruction only twofold at pH 4.28, but threefold at 5.2, and again only twofold at pH 7.9. It is more probable that the apparent destruction in 1 hour at pH 4.28 and at pH 5.2 is somewhat above the truth in the first instance (pH 4.28) and somewhat below the truth in the second (pH 5.2).

Allowing for the limitations of the method, it may be said that the destruction of vitamin B in 4 hours at 100° is measurable at pH 4.28, measurably greater at pH 5.2, and very markedly greater at pH 7.9. About the same amount of destruction of vitamin B occurred in 1 hour at pH 7.9 as in 4 hours at pH 5.2, and in 1 hour at pH 9.2 as in 4 hours at pH 7.9; while the destruction occurring in 1 hour was about twice as great at pH 9.2 as at pH 7.9, and in 4 hours was about twice as great at pH 7.9 as at pH 5.2.

Bringing all these data into one generalization it might be said that vitamin B is destroyed more rapidly at pH 5.2 than at pH 4.28 but that here the absolute rates of destruction are not high enough for satisfactory measurement of the ratio existing between them; the destruction is two to four times as rapid at pH 7.9 as at pH 5.2; and again it is two to four times as rapid at 9.2 as at 7.9.

By comparing these results with those of Sherman and Grose<sup>2</sup> we find that a reduction of the acidity of the medium from pH 4.28 to pH 5.2 increased the rate of destruction of vitamin B almost as much as did an increase of temperature from 100° to 110°C.; and that the destructive effect of changing the reaction from pH 4.28 to pH 7.9 was greater than that of increasing the temperature from 100° to 130°C.

This destruction of vitamin B appears not to be due in any appreciable degree to oxidation, for substantially identical results were obtained whether the heating was done in loosely covered flasks or under very strictly anaerobic conditions. This was found true both at natural acidity (pH 4.28) and in a solution sufficiently alkaline to result in the destruction of over half of the vitamin B in the experimental heating of 4 hours. The latter case would seem to be particularly well suited to test the question, and here also the destruction was apparently unchanged when all air and dissolved oxygen were very rigidly excluded.

#### SUMMARY AND CONCLUSIONS.

The extent of the destruction of vitamin B, upon heating for 1 hour and for 4 hours at 100°C., was measured by means of over 200 quantitative feeding experiments.

In most cases the direct comparison was between the vitamin B contents of solutions, one of which had been heated at the natural acidity of tomato juice (pH 4.28) and the other after shifting the pH by addition of alkali and then determining it by means of the electrometric method. Thus the change in rate of destruction which results from the change of pH of the medium was established directly by the experiments.

Similar comparisons have also been made between the solution (filtered tomato juice) heated at its natural acidity and parallel portions which were unheated. Hence results can be stated both in terms of the approximate percentage of vitamin B which was destroyed at each pH (as in Table I), and in terms of the directly measured changes resulting from the shifting of the pH as in the actual performance of the experiments in which the pH was thus changed.

The *increased* destruction of vitamin B *over that at* pH 4.28 (natural acidity of clear filtered tomato juice), on heating for 1

hour at 100°C. at pH 7.9 was 20 to 30 per cent; at pH 9.2, 60 to 70 per cent; at pH 10.9, 90 to 100 per cent of the total amount of vitamin B present.

When the solutions were heated for 4 hours at the same temperature, the increased destruction over that occurring at pH 4.28 was at pH 5.2, 10 to 20 per cent; at pH 7.9, 60 to 70 per cent.

Of the total initial amount of vitamin B, the percentages destroyed *by 1 hour of heating at 100°C.*, were as follows: at pH 5.2 about 10 per cent; at pH 7.9, 30 to 40 per cent; at pH 9.2, 60 to 70 per cent; at pH 10.9, 90 to 100 per cent.

The corresponding percentages destroyed *by 4 hours of heating at 100°C.* were: at pH 4.28, about 20 per cent; at pH 5.2, about 31 per cent; at pH 7.9, about 70 per cent.

The decrease of acidity from pH 4.28 to pH 5.2 increased the rate of destruction of vitamin B in these experiments to about the same extent as did the increase of temperature from 100° to 110°C. in the experiments of Sherman and Grose; and a change from pH 4.28 to pH 7.9 accelerated the destruction in greater degree than did a change of temperature from 100° to 130°C.

The rate of destruction of vitamin B at 100°C. was distinctly increased by shifting the pH of the solution from 4.28 to 5.2 although in this case the actual rates of destruction were too small to permit of satisfactory quantitative measurement of their ratio; at pH 7.9 the destruction was from two to four times more rapid than at pH 5.2; and at pH 9.2 the destruction was from two to four times more rapid than at pH 7.9

In these experiments, oxidation does not seem to have played any appreciable part in the observed destruction of vitamin B. It is more probable that the vitamin was destroyed either by an hydrolysis or by intramolecular rearrangement, and that in either case the destruction reaction was catalyzed by hydroxyl ions. Change in the ratio of hydrogen to hydroxyl ions influences the rate of destruction in the same manner whether the pH of the solution were on the acid or on the alkaline side of neutrality; *i.e.*, the rate of destruction increased both by reducing the acidity of an acid medium, and by increasing the alkalinity when the medium had already been made alkaline. In other words, on both sides of neutrality, the rate of destruction of the vitamin was a function of the pH of the medium in which it was dissolved.



## THE EFFECT OF THE ADMINISTRATION OF COD LIVER OIL UPON THYROPARATHYROIDECTOMIZED DOGS.

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Tetany, which is caused by or associated with defective calcium metabolism, very often accompanies rickets, a disease also characterized by faulty calcium and phosphorus utilization. Within the past few years numerous attempts have been made to cure infantile tetany by the use of methods known to have a beneficial action in the prevention and cure of rickets. Shortly after Huld-schinsky (1) reported that ultra-violet radiations cured rickets, Sachs (2) claimed that the symptoms of latent tetany could be alleviated by artificial sunlight. This claim was soon supported by Huld-schinsky (3) who reported that he had been able to cure manifest tetany in several infants by irradiating them with ultra-violet light. Following these investigations, Casparis and Kramer (4), Hoag (5), Lestocquoy (6), and Block and Faber (7) also showed that ultra-violet light has a therapeutic action in the treatment of tetany. Hoag (5) and Liu (8) reported the same for cod liver oil although the former obtained more marked effects with ultra-violet radiations.

While our knowledge concerning the effect of antirachitic agents upon infantile tetany has thus been increased, little is known of their influence upon experimental tetany produced by the removal of the parathyroid glands from dogs. It was for the purpose of studying this subject that the experiments described here were planned. However, while the work was in progress a paper appeared by Swingle and Reinhold (9) in which they gave the results of the treatment of ten parathyroidectomized dogs with ultra-violet light. They exposed their animals for varying lengths of time both before and after the removal of the glands. They were



TABLE I.  
*Effect of Administration of Cod Liver Oil upon Prevention of Tetany, Duration of Life, and Concentration of Serum Calcium.*

Dog No.	Treatment.		Days after operation.		Serum Ca.  <i>mg. per 100 cc.</i>	Remarks.
	Before operation.	After operation.	Before tetany.	Before death.		
16	Basal ration for 14 days.	None.	No tetany observed.	3+	(1 day.)* 10.07	Died during night.
17	Basal ration for 14 days.	"	2	4+	(1 day.) (4 days.) 8.33 5.91	" "
6	None.	20 cc. cod liver oil daily.†	2	2+	(2 days.) 6.62	" "
7	"	" "†	No tetany observed.	2+	(2 days.) 7.90	" "
21	Basal ration for 15 days.	"	3	3	(3 days.) 8.62	
9	Basal ration + 10 cc. cod liver oil daily for 13 days.	10 cc. cod liver oil daily.†	No tetany.	Discontinued.	(3 days.) 10.06	

15	Basal ration + 20 cc. cod liver oil daily for 13 days.	20 cc. cod liver oil daily.	"	21	(1 day.) 8.62	(5 days.) 7.55	(14 days.) 5.72	
20	Basal ration + 20 cc. cod liver oil daily for 14 days.	None.	"	54	(3 days.) 8.62	(13 days.) 6.22	(21 days.) 5.20	Fed meat on 2 successive days. No tetany.
24	Basal ration + 20 cc. cod liver oil daily for 13 days.	"	"	49	(2 days.) 7.94	(14 days.) 6.58	(29 days.) 4.85	Exercised on 29th day. No tetany. Died during night.
25	Basal ration + 20 cc. cod liver oil daily for 7 days.	"	4	21	(2 days.) 7.94	(14 days.) 5.04		Died during night. Slight tetany observed only once.
26	Basal ration + 20 cc. cod liver oil daily for 7 days.	"	6	17	(2 days.) 6.78	(6 days.) 4.84		Tetany observed only once while blood sample was being taken. Died during night.

\* Number of days after operation when blood sample was taken.

† Irradiated 20 minutes daily.

able to increase considerably the length of time that their animals lived after the operation. The average for their ten dogs was about 11 days instead of the usual 2 to 5 days. They found that although the animals' lives were lengthened the concentration of calcium in the blood fell, within 2 or 3 days, from 10 or 11 mg. per 100 cc. of serum to 6 or 7 mg. with the simultaneous onset of tetany. They concluded that the increased length of life of their animals was due to the treatment after the glands had been removed.

The following report deals with the results of feeding cod liver oil although the effect of ultra-violet irradiation was studied at the same time. A few animals were given both treatments and are included in this series of experiments.

#### EXPERIMENTAL.

Dogs were used as experimental animals. They were fed a basal ration of equal parts of autoclaved rolled oats and corn-meal supplemented with 5 gm. of casein and 2 gm. of sodium chloride daily. The rolled oats and corn-meal mixture was fed in such quantities that the animals could eat of it *ad libitum*. The cod liver oil was fed directly into the mouth of the animals from a graduated cylinder. To minimize the possibility of incomplete removal of the parathyroid glands the thyroids were extirpated with the parathyroids. Even under these conditions a certain number of animals fail to show symptoms due, presumably, to the presence of accessory parathyroid tissue.

Blood samples were taken by heart puncture at the time of operation and at varying intervals thereafter until the experiment was discontinued. The sera were analyzed for calcium. Most of the determinations were done by the method of deWaard (10). A few were done by Clark and Collip's (11) modification of Tisdall's method which apparently gives slightly lower and more consistent results.

After the removal of the glands the dogs were watched very closely for signs of tetany. In every case the dogs were continued on the basal ration although with a few exceptions they ate very little after the 2nd or 3rd day following the operation. A study was made of the effect of administering the cod liver oil before, as well as after, the removal of the glands. The influence of cod liver

oil upon the prevention of tetany, the duration of life, and the concentration of serum calcium is summarized in Table I, and greater details are given in the protocols appearing at the end of the paper.

From the data it is evident that cod liver oil is without apparent effect if administered only after the removal of the glands. On the other hand if the oil is given before and after the operation, or only before, it exerts a definite influence in prolonging life and preventing tetany. After the removal of the glands the average length of life of the untreated animals and of those which received the treatment only after the operation was about 3 days; whereas the average for the animals which were treated before operation (including Dog 15) was 32 days.

The control animals, and those which received treatment only after the operation, developed tetany within about 2 days. Dogs 7 and 16 were not observed in active tetany, but it will be noted, however, that both these animals died during the night. As death frequently follows within a short time after the first outward signs of tetany, these animals probably experienced such a condition before death. Of the animals which received treatment only before the removal of the glands, three were never seen to show the least signs of tetany although they were observed very closely throughout the entire experiments which lasted on an average of 41 days. Each of the two animals which received the cod liver oil for only 7 days before operation showed slight signs of tetany on one occasion only.

Although the animals which received treatment before operation had little or no tetany, all but Dog 20 showed very definite and quite similar signs of failure. For a few days following the operation they appeared perfectly normal. However, it was not long before they began to eat less and finally stopped eating entirely. The loss of appetite was accompanied by loss of weight and a greatly decreased activity on the part of the animals. Finally the dogs became very much emaciated. This condition was soon followed by coma and death. These symptoms are very similar to those observed by Thompson and Leighton (12) when they ligated the parathyroid glands in dogs. They summarize their results as follows:

"After ligation of all parathyroid tissue the dog passes the time limits of tetanic death that occurs after excision of the glandules, practically with-

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out symptoms. Gradually, however, chronic symptoms, trophic in nature, arise. These consist in gradual but progressive loss of weight and strength, greatly diminished resistance to infection, and a final stuporous condition ending in death without tetany."

The decrease in activity of the animals usually accompanied the loss of appetite. However, Dog 24 remained active for a number of days after it had stopped eating. Dog 20 showed none of these symptoms although the concentration of calcium fell as low as 5.20 mg. per 100 cc. of serum. This animal died very suddenly on the 54th day after operation when a sample of blood was being taken from the heart.

The fact that these animals showed little or no tetany is still more remarkable when the concentration of calcium in the blood is taken into consideration. It will be observed from the accompanying table that the cod liver oil was not efficacious in maintaining normal calcium levels. Not only did the concentration in the serum of the animals receiving treatment before operation fall as rapidly as that of the controls (and animals treated only after the removal of the glands) but it fell to even lower levels before the dogs died.

Some investigators believe that tetany is associated with excessive protein metabolism. Berkeley and Beebe (13), Marine (14), Paton and Findlay (15), and Sinelnikoff (16) all report that the symptoms of tetany are aggravated by the feeding of flesh. The latter worker was able to bring on attacks of active tetany in parathyroidectomized dogs by feeding from 400 to 800 gm. of raw meat. In view of these experiments Dog 20 was fed 1 pound of raw lean beef on the 28th day following the removal of the glands. A similar amount was given again on the following day. The dog ate the meat ravenously, but no signs of tetany developed.

It has often been observed that tetany may be made manifest by disturbing the animal. Consequently on the 29th day following the operation Dog 24 was taken from the cage, and in addition to the disturbance of taking a sample of blood, it was caused to run about the room. Although analyses on that day showed the concentration of calcium to be 4.85 mg. per 100 cc. of serum no tetany developed.

The cause of tetany has been the subject of much discussion but none of the various theories advanced has been universally ac-

cepted. Since MacCallum and Voegtlin (17) first showed that the concentration of calcium in the blood is greatly reduced during tetany, many have believed tetany to be due to the low calcium concentration *per se*. One of the most recent advocates of this theory is Collip (18) who has demonstrated that it is not only possible to maintain a normal calcium level and prevent tetany in parathyroidectomized dogs by the injection of the active principle of the parathyroid glands, but if the extract which he prepared is injected into a normal dog the calcium concentration is raised considerably above the ordinary level.

The low calcium theory, however, is not adhered to by all investigators. Paton and Findlay (19) were able to produce a condition of hyperirritability in cats, rats, and rabbits associated with convulsions very similar to parathyroid tetany by the injection of guanidine and methyl guanidine. Burns and Sharpe (20) also reported an increased excretion of guanidine and methyl guanidine in the urine of dogs following the removal of the parathyroid glands. However, the uncertainty concerning the accuracy of the quantitative method used makes the observations of doubtful value.

Tetany has also been observed under various conditions when the calcium was either normal or slightly reduced. Graham (21) states that he has observed cases of infantile tetany in which the calcium of the blood was normal. Steenbock, Jones, and Hart (22) noted tetany in dogs with rickets. In regard to the relation of the concentration of calcium to tetany they make the following remarks.

"It is difficult to say what significance is to be attached to the lowered Ca values. Both of the animals were observed at intervals in severe tetanic convulsions, but we have also observed this in dogs afflicted with rickets which had a normal calcium content of the blood. Apparently, a decreased calcium content cannot *per se* be the cause of the convulsions in these animals."

While these cases to which reference has just been made show that it is possible to have tetany with a normal concentration of calcium in the serum the data reported in the present paper show that it is also possible to have a greatly lowered concentration of calcium in the blood and still not have tetany. These experiments add to the data already accumulated which strongly suggest

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that factors other than the low concentration of serum calcium may influence the onset of parathyroid tetany.

Although no beneficial results were obtained if the oil were administered only after the removal of the parathyroid glands, these experiments do not prove that the presence of the glands is necessary for cod liver oil to exert its influence effectively. It appears that the feeding of 20 cc. of oil for 7 days is on the borderline of prevention and non-prevention of tetany. As none of the dogs which received the treatment only after the operation lived more than 3 days there is the possibility that the animals died before the oil could become effective. However, Block and Faber (7) believe that ultra-violet light acts in preventing tetany by stimulating the parathyroid glands to greater activity, which also may be true of cod liver oil.

### SUMMARY.

The daily administration of 20 cc. of cod liver oil for 2 weeks before operation, prevented tetany and greatly increased the length of life of thyroparathyroidectomized dogs. However, 20 cc. per day of the oil, if given only after the removal of the glands, had no effect in preventing tetany or in prolonging the life of the animals.

The concentration of calcium in the blood of the treated animals fell as rapidly as that of the untreated animals, and, in fact, the lowest levels attained were in the blood of the treated dogs which lived the longest. These data indicate that some factor in addition to the low concentration of serum calcium plays a part in the production of the tetany which usually follows, within 2 or 3 days, the removal of the parathyroid glands.

Similar results, but not as striking, were obtained by irradiating the dogs with ultra-violet light before the removal of the glands.

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## PROTOCOLS.

*Series I. Controls.*

- Dog 16, female; weight 12.2 kilos. No treatment.  
 Nov. 20, 1925. Began feeding basal ration.  
 Dec. 4. Thyroparathyroidectomy performed.  
 Dec. 5-6. Dog lively. Eating well.  
 Dec. 7. Dog quieter.  
 Dec. 8. Animal found dead in the morning.  
 Dog 17, male; weight 11.0 kilos. No treatment.  
 Nov. 20, 1925. Began feeding basal ration.  
 Dec. 4. Thyroparathyroidectomy performed.  
 Dec. 5. Dog normal.  
 Dec. 6-7. Dog quiet. Not eating.  
 Dec. 8. Violent tremors and tetany.  
 Dec. 9. Animal found dead in the morning.



## 656 Cod Liver Oil and Parathyroid Tetany

### *Series II. Treatment Only after Removal of Glands.*

Dog 6, male; not weighed. Cod liver oil and irradiation.

Feb. 5, 1925. Thyroparathyroidectomy performed at 5.30 p.m.

Feb. 6. Began giving 20 cc. of cod liver oil and 20 minutes of ultra-violet light treatment.

Feb. 7. A.m., slight trembling of facial muscles; p.m., dog in severe tetany.

Feb. 8. Animal found dead in the morning.

Dog 7, female; not weighed. Cod liver oil and irradiation.

Feb. 12, 1925. Thyroid and parathyroid glands removed. Began giving 20 cc. of cod liver oil and 20 minutes of ultra-violet light treatment daily.

Feb. 13. Dog very quiet. Not eating.

Feb. 14. Dog breathing with difficulty. Tremors.

Feb. 15. Animal found dead in the morning.

Dog 21, female; weight 11.6 kilos. Cod liver oil.

Dec. 31, 1925. Began feeding basal ration.

Jan. 15, 1926. Thyroparathyroidectomy performed. Began giving 20 cc. of cod liver oil.

Jan. 16. Dog active. Eating well.

Jan. 17. Dog active. Not eating very much.

Jan. 18. Severe tetany. Animal died in the late afternoon.

### *Series III. Treatment before and after Removal of Glands.*

Dog 9, male; weight 13 kilos. Cod liver oil and irradiation.

May 1, 1925. Began feeding basal ration. Also began treatment of 20 minutes irradiation and 10 cc. of cod liver oil daily.

May 12. Thyroparathyroidectomy performed.

May 13-14. Dog active. Eating well.

May 15-26. Dog active and eating very well. Experiment discontinued as blood calcium is still high, indicating that parathyroid tissue was not completely removed.

Dog 15, male; weight 13.2 kilos. Cod liver oil.

Nov. 20, 1925. Began feeding basal ration and 20 cc. of cod liver oil daily.

Dec. 4. Thyroid and parathyroid glands removed.

Dec. 5-7. Dog active. Eats well.

Dec. 8. Dog somewhat quieter and appetite slightly reduced.

Dec. 14. Animal has become very quiet. Does not eat. Both eyes sore.

Dec. 15-24. Not much change.

Dec. 25. Dog dead. No change from above. At no time has dog shown tetany.

### *Series IV. Treatment Only before Removal of Glands.*

Dog 20, male; weight 11.8 kilos. Cod liver oil.

Jan. 1, 1926. Began feeding basal ration and 20 cc. of cod liver oil.

Jan. 15. Thyroid and parathyroid glands extirpated. Discontinued cod liver oil feeding.

- Jan. 16-Feb. 11. Dog very active, eating well. Appears perfectly normal.  
Feb. 12. Dog given 1 pound of lean meat. No signs of tetany.  
Feb. 13. Dog given another pound of meat. No tetany.  
Feb. 14. Continued on basal ration.  
Feb. 15-Mar. 9. Dog very active. Eats well. Appears perfectly normal.  
Mar. 10. Died very suddenly while taking a sample of blood. At no time did it show any abnormal signs.  
Dog 24, male; weight 12.8 kilos. Cod liver oil.  
Feb. 20, 1926. Began feeding basal ration and 20 cc. of cod liver oil daily.  
Mar. 5. Thyroparathyroidectomy performed. Discontinued giving cod liver oil.  
Mar. 6. Dog active. Eats well.  
Mar. 7. Dog quieter. Appetite decreased.  
Mar. 8. Right eye sore.  
Mar. 9. No change.  
Mar. 10. Eyes clear. Dog more active. Not eating very much.  
Mar. 11-Apr. 2. Dog active but not eating.  
• Apr. 3. Dog taken out of cage and caused to run about room. No sign of tetany.  
Apr. 4-22. Dog gradually becoming very thin and weak.  
Apr. 23. Dog cannot stand.  
Apr. 24. Dog found dead in the morning.  
Dog. 25, male; weight 13.6 kilos.  
Feb. 26, 1926. Began feeding basal ration and 20 cc. of cod liver oil daily.  
Mar. 2. Thyroids and parathyroids removed. Discontinued giving cod liver oil.  
Mar. 6-8. Animal normal. Eats well.  
Mar. 9. Slight tetany.  
Mar. 10-14. Dog active. Appetite decreasing.  
Mar. 15. Dog becoming less active. Not eating.  
Mar. 16-25. Dog quiet and groans considerably. Does not eat.  
Mar. 26. Dog found dead in the morning. No tetany observed except on Mar. 9.  
Dog 26, female; weight 14.5 kilos. Cod liver oil.  
Mar. 27, 1926. Began feeding basal ration and 20 cc. of cod liver oil daily.  
Apr. 3. Thyroids and parathyroids extirpated.  
Apr. 4-5. Animal normal.  
Apr. 6-8. Not eating.  
Apr. 9. Slight tetany while blood sample was being taken.  
Apr. 10-19. No tetany. Dog very quiet. Not eating.  
Apr. 20. Animal died during night.



## ALCAPTONURIA IN A RABBIT.

By JULIAN H. LEWIS.

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(Received for publication, August 23, 1926.)

Alcaptonuria is a very rare disease of metabolism, first described by von Bödeker<sup>1</sup> in 1859. It is characterized by the production of a urine which turns to a dark brown or black color when exposed to the air. Chemically such a urine has a number of distinct characteristics. With the aid of heat it will reduce an alkaline solution of copper sulfate. Ammoniacal solutions of silver nitrate are reduced in the cold. With dilute solutions of ferric chloride a transitory blue color is produced. Millon's reagent when added to alcaptonuric urine forms an orange colored precipitate. Baumann and Wolkow<sup>2</sup> first demonstrated that the peculiarities of alcaptonuric urine are due to the presence of homogentisic acid (dihydroxyphenylacetic acid). Homogentisic acid can be isolated by several methods in large amounts and in pure condition from such urines. Just what the origin of homogentisic acid is, or what anomaly in the metabolic processes it represents, is not clear, but the results of experimental studies indicate that it results from a disturbance of the catabolism of the amino acids, particularly tyrosine and phenylalanine, in such a way that the final cleavage of the benzene ring beyond the stage represented by homogentisic acid is no longer possible. Tyrosine and phenylalanine when fed to an alcaptonuric individual appear in the urine as homogentisic acid. Homogentisic acid is readily burned in the normal individual but in the alcaptonuric it is excreted in the urine unchanged.

Garrod<sup>3</sup> has pointed out that the disease is congenital and

<sup>1</sup> von Bödeker, *Z. rationelle Med.*, 1859, vii, 130; *Ann. Chem. u. Pharm.*, 1861, cxvii, 98.

<sup>2</sup> Baumann, E., and Wolkow, M., *Z. physiol. Chem.*, 1891, xv, 228.

<sup>3</sup> Garrod, A. E., *Inborn errors of metabolism*, Oxford Medical Publications, 1909.

inheritable. It appears most often in females and has a tendency to occur in the children of parents who were first cousins. People with the disease appear otherwise to enjoy good health. Occasionally there is a deposit of black pigment in the cartilages of the body and in the capsules of the joints, a condition known as ochronosis. The association of alcaptonuria with ochronosis is not constant; ochronosis may occur without alcaptonuria, and conversely.

Up to the present time 50 to 60 cases of alcaptonuria have been reported in the literature, all of which have been in man. In animals (horses, cows, hogs, asses) a form of ochronosis occurs, but this condition is in no way related to ochronosis and alcaptonuria which occur in man. The pigmentation occurs in the bones of the animals and not in the cartilages, and the pigment appears to be a blood pigment.

A case of alcaptonuria in a rabbit has come to my attention which seems to be the only one that has been reported in a lower animal. On an occasion the animal keeper reported to me that a rabbit, which had been used for some time for immunological purposes, always produced a black tarry appearing soilage in its cage, entirely different from that produced by the other rabbits. The animal was a large white female rabbit apparently in good health. It was immediately isolated in a metabolism cage and the urine collected. The urine found in the bottle was an inky black fluid, free from any other pathological constituent that could be detected microscopically. The color was at first thought to be derived from contamination with feces which might have contained the products of an upper gastrointestinal hemorrhage, but the feces were found, on isolation, to be normal and to contain no traces of blood. A specimen of urine obtained from the bladder by catheterization had the appearance of normal rabbit urine, but when exposed to the air for a short time it became very dark. A portion of the fresh urine placed in a test-tube and covered with liquid paraffin retained its original normal appearance for over 24 hours. This urine reduced Fehling's solution very readily and gave a precipitate with ammoniacal silver nitrate without heating. When added to a solution of ferric chloride a blue color was produced which quickly disappeared. Millon's reagent gave a bulky red precipitate. It was concluded from these reactions that this was undoubtedly a case of true alcaptonuria.

As alcaptonuria is an inherited disease it was thought that this case presented an excellent opportunity to study experimentally the mechanism of its inheritance. Inherited metabolic disorders have never been investigated in this manner as they have been observed only in human subjects. Therefore, without further experimentation, this rabbit was placed in good living quarters and provided with a mate. However, the animal bore no young and, after a period of 3 or 4 months, died. On autopsy it was found that the entire system of generative organs was involved in a huge pelvic abscess and all the organs completely destroyed. A thorough search was made for other pathological conditions, especially for black deposits in the cartilages and in the capsules of joints such as occur in the ochronosis associated with alcaptonuria in the human. None was found, however.

About 2 liters of the black urine were preserved with toluene for the purpose of isolating homogentisic acid chemically. This was done without knowledge of the oxidative action of alkalies on homogentisic acid. When the chemical analysis was begun, after the death of the animal, the urine was found to be strongly alkaline. This was probably because the toluene failed to inhibit bacterial growth completely. In addition, rabbit urine is normally alkaline. The usual methods for isolating homogentisic acid failed to yield any of this substance. A black substance present in the preserved urine probably represented hydroquinones, the oxidative products of homogentisic acid.

With the exception of the actual isolation of homogentisic acid, this rabbit presented every feature characteristic of true alcaptonuria as found in man, and, as far as can be ascertained, is the only case of true alcaptonuria observed in a lower animal.



# THE CHEMICAL INVESTIGATIONS OF CORPUS LUTEUM.

## VI. THE LIPOIDS OF THE ETHER EXTRACT.

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(Received for publication, July 30, 1926.)

Modern work has clearly demonstrated the varying nature of lecithins isolated from different sources. Levene<sup>1-4</sup> and his collaborators found palmitic, stearic, oleic, and arachidonic acids in liver and brain lecithins.<sup>1</sup> In egg lecithin they found, in addition to these acids, linolic.<sup>5,6</sup> The egg lecithin differed also from the liver lecithin in containing a smaller proportion of the forms with the highly unsaturated fatty acids. Lecithins from the same source may also vary in their nature as McCollum, Halpin, and Drescher<sup>7</sup> showed in the case of the fatty acids of the egg yolk, which they demonstrated varied with the diet. Consequently in corpus luteum we might reasonably expect a different mixture of lecithins to be present than those found in the above sources.

From the ether extract of corpus luteum we were able to prepare a mixture of lecithins practically free from contamination with cephalin. On hydrolysis this gave evidence of the presence of palmitic and arachidonic acids and indications also of a tri unsaturated acid of the C<sub>20</sub> series. Oleic acid is probably also present. No indications of the presence of linolic acid were found. Stearic acid is probably also absent. Evidence for the presence of this new tri unsaturated acid of the C<sub>20</sub> series has

<sup>1</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlviii, 185.

<sup>2</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1922, li, 285.

<sup>3</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlv, 353.

<sup>4</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, liv, 99.

<sup>5</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlv, 193.

<sup>6</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 507.

<sup>7</sup> McCollum, E. V., Halpin, J. G., and Drescher, A. H., *J. Biol. Chem.*, 1912-13, xiii, 219.



also been found by us<sup>8</sup> in the neutral fat from the acetone extract of corpus luteum. Wesson<sup>9</sup> suggests the hypothesis that in the metabolism of at least an appreciable proportion of the fatty acids they are built up to the 20 carbon tetra unsaturated arachidonic acid. The finding of a tri unsaturated acid of the C<sub>20</sub> series in the lecithin complex from corpus luteum suggests the possibility that the arachidonic is formed by the desaturation of more saturated derivatives. The finding of Bull<sup>10</sup> of a 20 carbon fatty acid of the oleic series in the liver of the cod is also suggestive in this connection. The function of the liver in rendering fatty acids more unsaturated is well known. Possibly this capacity for the reduction of fatty acids is also an important function of the corpus luteum and other glands rich in lipoids.

Our ether extract of corpus luteum was made on material that had previously been extracted with acetone. The results of the examination of the acetone extract have been reported by us in previous publications<sup>8,11-13</sup> from this laboratory. In this ether extract of corpus luteum we were able to show traces of protagon-like material. This probably represents contamination of ovarian residue in the commercial corpus luteum employed as we have found<sup>14</sup> large quantities of this ether-insoluble material in ovarian residue.

We were interested not only in the chemistry of the commercial corpus luteum but also in the question as to how the lecithin and cephalin fractions prepared from different lots of corpus luteum varied. Two different lots of corpus luteum were used and lecithin and cephalin fractions prepared by identical methods. The lecithin fractions varied but little but there was considerable variation in the cephalin fraction, one of them containing considerably more nitrogen than the other.

After we have studied the chemical composition of the commercial ovarian preparations it is intended in this laboratory to

<sup>8</sup> Cartland, G. F., and Hart, M. C., *J. Biol. Chem.*, 1925, lxvi, 619.

<sup>9</sup> Wesson, L. G., *J. Biol. Chem.*, 1925, lxv, 235.

<sup>10</sup> Bull, H., *Ber. chem. Ges.*, 1906, xxxix, 3570.

<sup>11</sup> Hart, M. C., and Heyl, F. W., *J. Am. Pharm. Assn.*, 1924, xiii, 17.

<sup>12</sup> Hart, M. C., and Heyl, F. W., *J. Biol. Chem.*, 1925, lxvi, 639.

<sup>13</sup> Hart, M. C., and Heyl, F. W., *J. Am. Pharm. Assn.*, 1925, xiv, 770.

<sup>14</sup> Unpublished work in this laboratory.

repeat this work on material representing different known stages of pregnancy.

#### EXPERIMENTAL.

Our samples represented carefully collected, hand-dissected material, dried immediately after collection below 40°C. in the commercial way. It is representative of the material found on the pharmaceutical market in that it has been taken from such a large number of cattle as to represent a good average sample. The material represented 6 parts of the fresh corpus luteum substance.

The material was ground to pass through a No. 20 sieve. Two lots of this material consisting of 4.073 kilos (Sample A) and 1 kilo (Sample B) were thoroughly extracted with acetone, freed from traces of this solvent in a vacuum drier, and then exhaustively extracted with purified ether. Four 25 liter portions of ether were used for Sample A and proportional amounts for Sample B.<sup>15</sup>

*Examination of the Ether Extract.*—The filtered ether extracts were concentrated<sup>16</sup> to 800 cc. An aliquot showed the presence of 259.7 gm. (6.38 per cent) in this extract. The solid material contained 3.13 per cent phosphorus and 2.81 per cent nitrogen. The ratio of nitrogen to phosphorus was therefore 1.99 to 1.

Nothing separated from this concentrated ether extract on standing for some time in the cold and it was precipitated by pouring it into 4 liters of pure ice-cold acetone. A slightly gummy amorphous precipitate was formed. This was centrifuged off and dissolved in 800 cc. of pure dry ether. This on standing at 0°C. for 48 hours separated some white insoluble material (1). The ether filtrate from (1) was precipitated by pouring into 3.5 liters of cold acetone. The amorphous precipi-

<sup>15</sup> In reporting the experimental results, only the details of the preparation of various fractions from Sample A will be reported. It will be understood though that similar fractions have been prepared in an analogous manner from Sample B, the results of analysis of which are reported along with those of Sample A.

<sup>16</sup> All distillations in this work were made under reduced pressure, at 40°C. or below in the presence of nitrogen. When solutions were allowed to stand they were saturated with nitrogen, stoppered, and allowed to remain in the ice box. Solids were dried in a black vacuum desiccator to prevent action of the light.

tate on being taken up again in 500 cc. of ether separated more insoluble material (1). The filtrate from this second insoluble material was again precipitated with 3 liters of acetone. The final ether solution separated no more ether-insoluble material on standing in the cold.

*Preparation of the Acetone- and Alcohol-Insoluble Lipoids (Crude Cephalin Fraction).*—The ether solution above was precipitated with 3 liters of ice-cold absolute alcohol. A slightly brown flocculent precipitate was formed. This was centrifuged off, dissolved in 400 cc. of pure dry ether, and cooled to 0°C. for 24 hours. Nothing separated from this ether solution and this process of precipitation with alcohol was repeated twice with 3 liters of cold alcohol, the final precipitation being from 300 cc. of ether and 2 liters of alcohol. The brown flocculent cephalin fraction was centrifuged off and dried to constant weight *in vacuo*. It weighed 46.3 gm. The cephalin fraction from Sample B was similar in appearance and weighed, dried *in vacuo*, 15.0 gm.

*Analyses.*

Sample A.	0.0683, 0.0672 gm. substance: cc. N/20 NaOH, 52.66, 51.41. 0.0988, 0.0996 " " : " N/50 NH <sub>3</sub> , 7.16, 7.27. Found. P 4.26, 4.23; N 2.03, 2.05.
Sample B.	0.0530, 0.0536 gm. substance: cc. N/20 NaOH, 37.53, 38.02. 0.0968, 0.0955 " " : " N/50 NH <sub>3</sub> , 10.98, 10.91. Found. P 3.92, 3.92; N 3.18, 3.20.
	Sample A. N:P::1.07:1. " B. N:P::1.80:1.

Prepared by similar methods there is considerable difference in the crude cephalins prepared from different lots of commercial corpus luteum.

*Examination of the Ether-Insoluble Material from the Acetone-Insoluble Lipoids (1).*—This material was a white flocculent solid. It was washed thrice with 50 cc. of ether and dried *in vacuo*. Weight 4.91 gm. It had no definite melting point, becoming gummy at 150°C. and softening to a vaseline-like consistency at 183–185°C. It contained nitrogen and phosphorus.

<i>Analyses.</i>	0.0967, 0.0987, gm. substance: cc. N/50 NH <sub>3</sub> , 14.87, 15.50. 0.1838, 0.1752 " " : " N/20 NaOH, 91.27, 87.05. Found. N 4.31, 4.40; P 2.75, 2.75. N:P::3.5:1.
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The physical properties of this material suggest its protagon-like nature. It was thoroughly extracted with ether and crystallized twice from 40 cc. of alcohol. Pure white, micro semi-crystalline ball-like material was obtained. Dried to constant weight *in vacuo* it weighed 0.364 gm. It softened at 180–185°C. and gave a positive orcinol test for cerebroside.

*Analyses.* 0.0619, 0.0607 gm. substance: cc. N/20 NaOH, 21.45, 21.20.  
0.0589, 0.0621 " " : " N/50 NH<sub>3</sub>, 6.90, 7.51.  
Found. P 1.91, 1.93; N 3.28, 3.39.  
N:P::3.8:1.

This material is of a protagon-like nature consisting of a mixture of sphingomyelin and cerebroside. This material is probably derived from ovarian substance contaminating the corpus luteum which we have found to yield large quantities of this ether-insoluble material.

*Examination of the Acetone-Ether Mother Liquors from the Precipitation of the Acetone-Insoluble Lipoids.*—These filtrates were taken to dryness, the residue dissolved in a small amount of ether, and a slight amount of acetone-insoluble material removed by pouring this into cold acetone. The acetone filtrate was then concentrated to dryness, the residue taken up in ether, and this process of precipitation repeated twice with acetone and twice with alcohol. Finally 300 cc. of an alcohol solution of ether, acetone, and alcohol-soluble material were obtained. This alcohol solution was precipitated with 500 cc. of a saturated alcoholic solution of cadmium chloride. A white flocculent precipitate was formed which was centrifuged off, washed thrice with 50 cc. of alcohol, and dried *in vacuo*. Weight 12.65 gm. 3.1 gm. of a similar compound were obtained from Sample B.

*Analyses.*

Sample A. 0.0616, 0.0634 gm. substance: cc. N/20 NaOH, 30.43, 31.10.  
0.1003 gm. substance: cc. N/50 NH<sub>3</sub>, 5.11.  
Found. P 2.74, 2.71; N 1.43.

Sample B. 0.0652, 0.0625 gm. substance: cc. N/20 NaOH, 29.74, 28.27.  
0.1004, 0.0995 " " : " N/50 NH<sub>3</sub>, 4.85, 4.74.  
Found. P 2.52, 2.50; N 1.35, 1.33.

Sample A. N:P::1.16:1.

" B. N:P::1.18:1.

Sample A and Sample B were joined for further purification. The combined cadmium chloride salts were moistened with 95 per cent alcohol and washed thrice with ether containing 2 per cent alcohol. The ether-insoluble material was decomposed in chloroform solution with methyl alcoholic ammonia solution by the method of Levene and Simms.<sup>1</sup> The chloroform alcoholic filtrate from the cadmium hydroxide gave on evaporation a vaseline-like residue of lecithin. This was thoroughly emulsified with 50 cc. of water in the presence of a trace of salt and precipitated in the cold with 20 cc. of acetone. This process of emulsification and precipitation was repeated twice and the final product dissolved in ether. On standing in the cold this ether solution separated a slight amount of insoluble material which was centrifuged off. The clear ether filtrate was poured into 250 cc. of cold absolute alcohol. Some flocculent material separated which was removed by centrifugation. The clear alcoholic filtrate was then precipitated with 200 cc. of a saturated alcoholic solution of cadmium chloride. A flocculent light tan-colored precipitate of the lecithin cadmium chloride formed. This was centrifuged off and washed thrice with 50 cc. of alcohol and once with 80 cc. of ether containing 2 per cent of alcohol. This was dried to constant weight *in vacuo*. Weight 4.946 gm. It was then crystallized from 125 cc. of a mixture of 2 parts of ethyl acetate and 1 part of 80 per cent ethyl alcohol. The light tan-colored microcrystalline lecithin cadmium chloride was centrifuged off and washed with the ethyl acetate-alcohol mixture and with ether. Dried to constant weight *in vacuo* it weighed 3.313 gm.

*Analyses.* 0.0688, 0.0683 gm. substance: cc. N/20 NaOH, 36.77, 36.39.  
0.0994, 0.0986 " " : " N/50 NH<sub>3</sub>, 5.11, 5.06.  
Found. P 2.96, 2.95; N 1.44, 1.44.  
N:P::1.08:1.

2.2891 gm. of this purified lecithin cadmium chloride compound were hydrolyzed by boiling for 12 hours with 100 cc. of 10 per cent hydrochloric acid. The mixture was cooled, filtered, concentrated, and nearly neutralized with 50 per cent sodium hydroxide. The solution was again concentrated, made alkaline with sodium hydroxide, then acid with acetic acid, filtered, and made up to a volume of 20 cc. 2 cc. samples were analyzed for

total nitrogen and amino nitrogen. 1.1 per cent of the total nitrogen was found in the amino form.

It is possible therefore to prepare a lecithin practically free from cephalin contamination from the ether-acetone filtrate from the acetone-insoluble material (ordinary lecithin and cephalin mixture).

*Examination of the Alcoholic Ether Mother Liquors from the Precipitation of Cephalin (Ordinary Lecithin Fraction).*—These solutions were joined and evaporated to dryness, the residue dissolved in 200 cc. of dry ether and treated with 1.5 liters of absolute alcohol. On standing in the cold some alcohol-insoluble material separated. This was centrifuged off and the filtrate evaporated to dryness again. This process of dissolving in ether and diluting with alcohol was repeated four times until the alcohol solution separated no more insoluble material on standing at 0°C. The final alcohol solution of 400 cc. was precipitated with an equal volume of alcoholic cadmium chloride solution. A heavy white precipitate of lecithin cadmium chloride was obtained. This precipitate was filtered off, washed thrice with 100 cc. of 95 per cent alcohol, and dried to constant weight *in vacuo*. Weight 42.47 gm. Sample B yielded 24.65 gm. of lecithin cadmium chloride at this point.

*Analyses.*

Sample A.	0.0610, 0.0621 gm. substance:	cc. N/20 NaOH, 31.85, 31.42.
	0.1048, 0.1061 " " :	" N/50 NH <sub>4</sub> , 6.05, 6.13.
	Found.	P 2.89, 2.80; N 1.62, 1.62.
Sample B.	0.0592, 0.0637 gm. substance:	cc. N/20 NaOH, 32.26, 34.87.
	0.1252, 0.1254 " " :	" N/50 NH <sub>4</sub> , 8.43, 8.19.
	Found.	P 3.01, 3.03; N 1.88, 1.83.
	Sample A.	N:P::1.26:1.
	" B.	N:P::1.36:1.

Each of these samples of lecithin cadmium chloride was put through the same process of purification, of emulsification, and crystallization described above, used in purifying the lecithin from the ether-acetone mother liquors from the precipitation of the acetone-insoluble lipoids. Sample A yielded 17.9 gm. of purified lecithin cadmium chloride salt and Sample B gave 10.4 gm.

*Analyses.*

Sample A.	0.0870, 0.0684 gm. substance:	cc. N/20 NaOH, 37.74, 38.45.
	0.0997, 0.0996 " " :	" N/50 NH <sub>4</sub> , 5.59, 5.54.
	Found.	P 3.11, 3.11; N 1.57, 1.56.

Sample B. 0.0639, 0.0681 gm. substance: cc. N/20 NaOH, 38.67, 38.18.  
 0.1000, 0.0995 " " : " N/50 NH<sub>3</sub>, 5.87, 5.68.  
 Found. P 3.10, 3.10; N 1.64, 1.60.  
 Sample A. N:P::1.11:1.  
 " B. N:P::1.16:1.

This process of emulsification of the lecithin and crystallization of the lecithin cadmium chloride salt from the ethyl acetate-alcohol mixture was repeated once more. This last step in the purification process had no great effect on the purity of these fractions as shown by the analyses below. Sample A yielded 6.43 gm. and Sample B 4.17 gm. of the purified lecithin cadmium chloride salt.

*Analyses.*

Sample A. 0.0684, 0.0690 gm. substance: cc. N/20 NaOH, 37.80, 38.46.  
 0.0987, 0.0998 " " : " N/50 NH<sub>3</sub>, 5.35, 5.62.  
 Found. P 3.06, 3.08; N 1.52, 1.58.  
 Sample B. 0.0675, 0.0675 gm. substance: cc. N/20 NaOH, 38.27, 38.27.  
 0.0985, 0.0979 " " : " N/50 NH<sub>3</sub>, 5.46, 5.67.  
 Found. P 3.13, 3.13; N 1.55, 1.62.  
 Sample A. N:P::1.12:1.  
 " B. N:P::1.12:1.

*Hydrolysis of the Lecithin Cadmium Chloride.*—9.7862 gm. of the mixed purified lecithin cadmium chloride salts from Sample A and Sample B were hydrolyzed by boiling under a reflux with 170 cc. of 10 per cent hydrochloric acid for 9 hours. The reaction mixture was cooled and the fatty acids extracted with ether. The washed and dried ether extract yielded on evaporation 4.63 gm. (47.3 per cent) of a brownish oil.

These fatty acids were separated by the barium-lead salt process of Levene.<sup>6</sup> This separation is based upon the fact that the barium salts of oleic acid and also of the saturated acids are insoluble in a mixture of benzene and ether. The barium salts of the more highly unsaturated acids are soluble in this mixture. Oleic acid is separated from the saturated acids by the solubility of its lead salt in ether.

By this means the fatty acids were separated into the following three fractions:

1. 1.979 gm. of saturated acids.
2. 0.689 gm. of intermediate fraction of the unsaturated acids (oleic).

## 3. 1.475 gm. of the more highly unsaturated acids.

*Examination of the Saturated Acids from Hydrolysis of the Lecithin Cadmium Chloride Compound.*—These were systematically fractionally crystallized five times into three fractions.

*Fraction I.*—Weight 0.4122 gm. This melted at 56–57°C. This was dried to constant weight *in vacuo* at the temperature of boiling toluene and analyzed.

*Analysis.* 0.1617 gm. substance: CO<sub>2</sub> 0.4451, H<sub>2</sub>O 0.1800.

Calculated for C<sub>18</sub>H<sub>32</sub>O<sub>2</sub>. C 75.0, H 12.5.

Found. " 75.1, " 12.5.

A mixed melting point with pure palmitic acid was not depressed. The melting point of this acid suggests a mixture of palmitic and stearic acids yet the mixed melting point and analyses indicate palmitic.

*Fraction II.*—Weight 0.4565 gm. This melted at 56–57°C. This was dried to constant weight *in vacuo* at the temperature of boiling toluene and analyzed.

*Analysis.* 0.1639 gm. substance: CO<sub>2</sub> 0.4497, H<sub>2</sub>O 0.1833.

Calculated for C<sub>18</sub>H<sub>32</sub>O<sub>2</sub>. C 75.0, H 12.5.

Found. " 74.8, " 12.5.

This fraction also analyzes for palmitic acid.

*Fraction III.*—This was a small fraction weighing 0.1784 gm. This was not further analyzed.

*Examination of Intermediate Fraction of Fatty Acids.*—This material had an iodine number of 98.2. The remainder of this solution was reduced with colloidal palladium by the method of Paal. A solid fatty acid was obtained which failed on crystallization from alcohol to give material of sufficient purity to warrant further analytical work. The properties of this fraction suggest oleic acid contaminated with a small amount of more highly unsaturated acids.

*Examination of the More Highly Unsaturated Fatty Acids.*—These acids had an iodine number of 126.3. The remainder of the material (1.361 gm.) was dissolved in 10 cc. of ether, cooled to 0°C., and brominated by the method of Baughman and Jamieson.<sup>17</sup> The bromine mixture was allowed to stand overnight at

<sup>17</sup> Baughman, W. F., and Jamieson, G. S., *J. Am. Chem. Soc.*, 1922, liv, 2947.



0°C. The insoluble bromides were centrifuged off and washed thrice with 7 cc. portions of cold absolute ether, and dried to constant weight *in vacuo*. This fraction was light gray in color and weighed 0.2026 gm. It decomposed at 225–227°C. (absence of linolenic hexabromide which melts at 183°C.). This fraction was separated by extraction for 8 hours in a Soxhlet apparatus into a benzene-insoluble and a benzene-soluble fraction.

*Fraction I (Benzene-Insoluble Material).*—This was a light gray powder weighing 0.110 gm. It decomposed sharply at 236–237°C. This was dried to constant weight *in vacuo* at the temperature of boiling toluene and the bromine determined by the Carius method.

*Analysis.* 0.0690 gm. substance: AgBr 0.1102.

Calculated for  $C_{20}H_{32}O_2Br_8$ . Br 67.8.

Found. " 67.9.

This material is arachidonic octobromide and it results from the bromination of the arachidonic acid present in the lecithin.

*Fraction II (Benzene-Soluble Material).*—On the evaporation of the benzene 0.089 gm. of a colorless substance was obtained. This blackened and decomposed sharply at 216–217°C.

*Analysis.* 0.0723 gm. substance: AgBr 0.1024.

Calculated for  $C_{20}H_{34}O_2Br_6$ . Br 61.0.

" "  $C_{18}H_{30}O_2Br_6$ . " 63.3.

Found. " 60.3.

The absence of material melting at 183°C. in these ether-insoluble bromides shows the absence of linolenic hexabromide. The analysis of this benzene-soluble, ether-insoluble bromide agrees fairly closely for that of a hexabromide of the  $C_{20}$  series. This indicates the presence, besides the well known arachidonic acid, of a tri unsaturated acid of the same series in the lecithin complex. Evidence of this new acid was also found by us<sup>8</sup> in the neutral fat in the acetone extract of corpus luteum. This makes another link in the chain of evidence, that in the metabolism of the fatty acids some of them might be built up to the  $C_{20}$  form and then desaturated and combined in the more labile lecithin complex before utilization by the body.

*Examination of the Petroleum Ether-Insoluble Bromides.*—The filtrate and washings from the ether-insoluble bromides were washed with sodium thiosulfate solution to remove excess bromine, the thiosulfate removed by water, and the ether solution dried and evaporated. A brownish oil weighing 2.42 gm. was obtained. On treating this oil with petroleic ether a resin and some semicrystalline material separated. The semicrystalline material weighed 0.0865 gm. and melted at 120–121°C. Systematic crystallization of this material from small amounts of alcohol and ether gave fractions melting very indefinitely at 130–150°C. to a fraction that softened at 155°C., gradually turning darker and decomposing at 220°C. The study of this fraction indicated the absence of linolic tetrabromide and the presence of traces of this hexa unsaturated acid of the C<sub>20</sub> series.

The resinous material referred to above weighed 0.4038 gm. This was all soluble in ether in the cold. Systematic study of this with the use of various solvents yielded only indefinite black gummy material from which nothing definite could be learned.

*Examination of the Petroleum Ether-Soluble Bromides.*—The petroleum ether was removed from this fraction. A light brown oil weighing 1.894 gm. was obtained. This was reduced with copper-coated zinc by the method of Wesson.<sup>18</sup> The alcohol solution of the debrominated acids was concentrated, water added, and extracted with ether. The washed and dried ether solution yielded on evaporation 0.4998 gm. of a yellow oil. This had an iodine number of 107. This iodine number is suggestive of the presence of oleic acid contaminated with slight amounts of more unsaturated acids.

*Examination of the Aqueous Hydrolysis Liquors from Hydrolysis of the Lecithin Cadmium Chloride Salt.*—These liquids were concentrated to a small volume, nearly neutralized with 25 per cent sodium hydroxide, filtered, the filtrate concentrated, made just alkaline with sodium hydroxide, then acid with acetic acid, and made up to volume. Total nitrogen and amino nitrogen determinations showed the presence of 2.7 per cent of the total nitrogen to be in the amino form. This indicates that the material hydrolyzed was practically free from contamination with cephalin.

<sup>18</sup> Wesson, L. G., *J. Biol. Chem.*, 1924, 1x, 183.

## SUMMARY.

The ether-soluble lipoids of corpus luteum have been studied with the following results.

1. Cephalin fractions prepared by identical methods from two different lots of corpus luteum varied widely in their nitrogen content.

2. Lecithins from two different lots of corpus luteum, prepared from the acetone-insoluble lipoids, agreed fairly closely in their analyses.

3. The lecithins from the acetone-insoluble lipoids were prepared practically free from cephalin contamination. These lecithins gave on hydrolysis evidence of the presence in their molecule of palmitic, arachidonic, oleic, and a new tri unsaturated acid of the  $C_{20}$  series. Linolenic and linolic acids appeared to be absent and the presence of stearic acid is doubtful.

4. The acetone-soluble fractions from two different lots of corpus luteum yielded lecithins closely alike in their analyses. These were prepared free from cephalin.

5. The ether-insoluble material yielded a small amount of material of a protagon-like nature. This material was probably derived from ovarian residue contamination of the commercial corpus luteum studied.

## THE CHEMICAL INVESTIGATIONS OF CORPUS LUTEUM.

### VII. THE CEPHALIN FRACTION.

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(Received for publication, July 30, 1926.)

According to Levene<sup>1</sup> the so called cephalin consists of true cephalin and of all the products of its intermediate hydrolysis; namely, of mono-fatty acid-glycerophosphoric aminoethanol ester, mono-fatty acid-glycerophosphoric acid, and glycerophosphoric acid. The acetone-insoluble and alcohol-insoluble fraction of the liver phosphatides was found by Levene<sup>2</sup> to be a mixture of little lecithin, little cephalin, and a variety of fragments of these and perhaps of other lipoids.

We were interested in the chemistry of the acetone- and alcohol-insoluble fractions from the ether extract of corpus luteum. This material was fractionated by the two methods for the purification of cephalin outlined by Levene.<sup>3</sup> Various fractions were obtained, the analysis of which indicated that the cephalin fraction of corpus luteum was similar in its nature chemically to the cephalin fractions obtained from the liver, heart, and brain.

Two different lots of commercial corpus luteum of 4.07 kilos (Sample A) and 1 kilo (Sample B) respectively were studied. Prepared by identical methods from two different lots of corpus luteum, cephalin fractions of widely different nitrogen and phosphorus content were prepared.

#### EXPERIMENTAL.

The cephalin fractions studied consisted of the acetone-insoluble lipoids from the ether extract of corpus luteum. All ether-insoluble

<sup>1</sup> Levene, P. A., and Komatsu, S., *J. Biol. Chem.*, 1919, xxxix, 91.

<sup>2</sup> Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1921, xliii, 359.

<sup>3</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, liv, 93.

and acetone-soluble material had been removed by repeated precipitation of the ether solution with acetone. The alcohol-soluble fraction (lecithin) had also been removed by repeated precipitation of the ether solution by alcohol.

The ether-soluble, acetone-, and alcohol-insoluble fraction from Sample A weighed 46.3 gm. It contained 2.04 per cent nitrogen and 4.25 per cent phosphorus. The nitrogen to phosphorus ratio was 1.07 to 1. The cephalin fraction from Sample B was 15.0 gm. in amount. This had a nitrogen and phosphorus content of 3.19 and 3.92 per cent respectively, with a nitrogen to phosphorus ratio of 1.80 to 1. Prepared by identical methods we thus had two samples of cephalin varying widely in their nitrogen content particularly.

45.6 gm. of the cephalin from Sample A were submitted to further purification using the first method for the purification of cephalin outlined by Levene and Rolf.<sup>3</sup> This material was dissolved in 160 cc. of warm glacial acetic acid, and allowed to stand 12 hours at 10°C. An insoluble fraction (1) was formed which was removed by the centrifuge and the clear filtrate was precipitated by pouring into 2 liters of alcohol. The amorphous precipitate (2) was separated by means of the centrifuge and the clear filtrate concentrated *in vacuo* to dryness. The vaseline-like residue was emulsified with 75 cc. of water and precipitated with 30 cc. of acetone at 0°C. The flocculent precipitate was centrifuged off, dissolved in 30 cc. of ether, and precipitated with 200 cc. of absolute alcohol. The brown flocculent precipitate was centrifuged off, and dried to constant weight *in vacuo*. This formed a light brown material that was easily ground up to a fine powder. Weight 6.965 gm. This was analyzed.

*Analyses.*

Sample A. 0.0781 gm. substance: cc. N/50 NH<sub>3</sub>, 5.06.

N 1.82.

0.0780, 0.0754 gm. substance: cc. N/20 NaOH, 52.77, 51.00.

P 3.74, 3.74.

N:P::1.07:1.

There was insufficient material for further purification. This material was then hydrolyzed to get the nitrogen distribution. 6.4251 gm. were hydrolyzed by boiling for 11 hours with 100 cc. of 10 per cent hydrochloric acid. The mixture was cooled and

extracted with ether thrice. The ether solution was washed, dried, and the ether removed. 4.017 gm. (62.5 per cent) of fatty acids were obtained. These fatty acids had an iodine number (Hanus) of 134, and contained 0.32 per cent nitrogen.

*Analysis.* 0.1106, 0.1030 gm. substance: cc. N/50  $\text{NH}_3$ , 1.26, 1.16.  
N 0.32, 0.32.

Theoretically cephalin should give 75.9 per cent of fatty acid. This low finding of fatty acids indicates that this fraction contains some of the fragments of the ordinary cephalin molecule with part of the fatty acids split off.

The aqueous hydrolysis liquors from the above material were concentrated to a small volume, nearly neutralized with 50 per cent sodium hydroxide, concentrated, made acid with acetic, filtered, and made up to a volume of 100 cc. Total nitrogen and amino nitrogen determinations showed that 83.7 per cent of the total nitrogen was in the amino form.

*Analyses.* 2.00, 2.00 cc. samples: cc. N/50  $\text{NH}_3$ , 6.94, 7.01.  
Total N 0.001946, 0.001964 gm., average 0.001952.  
1.50, 1.50 cc. sample: cc. N at 28°C. and 734 mm. pressure;  
2.43, 2.41.  
Blanks: cc. N at 28°C. and 734 mm. pressure, average 0.12 cc.  
Average  $\text{NH}_2$  N from 2.00 cc., 3.11 cc., 0.001635 gm.  
$$\frac{0.001635}{0.001952} = 83.7 \text{ per cent of the total nitrogen in the amino form.}$$

The same process of purification applied to cephalin from Sample B gave 1.868 gm. of material. This analyzed as follows:

*Analyses.* 0.1004, 0.0984 gm. substance: cc. N/50  $\text{NH}_3$ , 8.27, 8.30.  
N 2.31, 2.36.  
0.0726, 0.0700 gm. substance: cc. N/20 NaOH, 45.00, 43.44.  
P 3.43, 3.43.  
N:P::1.51:1.

*Examination of Glacial Acetic Acid-Insoluble Material (1).—*  
This insoluble material from both samples of corpus luteum weighed 8.53 gm. Of this 7.53 gm. were ether-soluble. 5.83 gm. of this were insoluble in hot alcohol. This material was purified twice by solution in 15 cc. of ether and precipitation with

100 cc. of alcohol. The light brown flocculent material was dried to constant weight *in vacuo*. Weight 2.995 gm.

*Analyses.* 0.0980, 0.0978 gm. substance: cc. N/50  $\text{NH}_3$ , 5.82, 5.82.  
 N 1.66, 1.67.  
 0.0700, 0.0684 gm. substance: cc. N/20 NaOH, 75.68, 73.80.  
 P 5.98, 5.97.  
 N:P::1:1.63.

2.0662 gm. of this material were hydrolyzed by boiling for 16 hours with 100 cc. of 10 per cent hydrochloric acid. The fatty acids recovered from an ether extract of the hydrolysis products weighed 1.0290 gm. (49.8 per cent). These acids had an iodine number of 125.

The aqueous solution was concentrated, made nearly alkaline with 50 per cent NaOH, concentrated, made acid with acetic acid, filtered, and made up to a volume of 50 cc. This was analyzed.

*Analyses.* 2.00, 2.00 cc. samples: cc. N/50  $\text{NH}_3$ , 4.26, 4.40.  
 Total N 0.00119, 0.00123 gm., average 0.00121 gm.  
 2.00, 2.00 cc. samples: cc. N at 27°C. and 745 mm. pressure, 2.14, 2.15.  
 Blanks: cc. N at 27°C. and 745 mm. pressure, average 0.14 cc.  
 Average  $\text{NH}_2\text{N}$  from 2.00 cc., 0.00107 gm.  

$$\frac{0.00107}{0.00121} = 88 \text{ per cent of the total nitrogen in the amino form.}$$

The analysis of this fraction indicates that it is a type of compound representing a deeper disintegration of the cephalin molecule than the acetic acid-soluble material. It differs from ordinary cephalin in containing a smaller proportion of the fatty acids and by a smaller proportion of the base aminoethanol. It is a good representative of the "fragment type" of compound that Levene has found to represent largely the chemistry of the so called cephalin or ether-soluble, acetone-, and alcohol-insoluble phosphatide fraction. In other words, this is further proof that the so called cephalin fraction of corpus luteum consists of true cephalin with the products of its intermediate hydrolysis; namely, mono-fatty acid-glycerophosphoric aminoethanol ester, mono-fatty acid-glycerophosphoric acid, and probably glycerophosphoric

acid. This glacial acetic acid-insoluble material from the cephalin fraction contained relatively small amounts of lecithin contamination.

*Examination of Insoluble Material Separating from Acetic Acid Solution on Dilution with Alcohol (2).*—This material from Sample A was dissolved in 100 cc. of ether, cooled to 0°C., and centrifuged from a little vaselin-like insoluble material. The ether solution was precipitated by pouring into 1.4 liters of alcohol. A brown flocculent precipitate was formed. This was centrifuged off, emulsified with 100 cc. of water in the presence of a trace of salt, cooled, and precipitated with 50 cc. of 10 per cent hydrochloric acid. This precipitate was centrifuged off and emulsified again with 175 cc. of water and precipitated in the cold with acetone. This emulsification process was repeated twice. The final product was dissolved in 75 cc. of ether and precipitated with 600 cc. of absolute alcohol. The brown flocculent precipitate was centrifuged off and dried to constant weight *in vacuo*. This fraction from Sample A weighed 13.23 gm. and that from Sample B 2.47 gm.

*Analyses.*

Sample A.	0.0638, 0.0618 gm. substance: cc. N/20 NaOH, 39.48, 38.56. P 3.42, 3.44. 0.0839, 0.0850 gm. substance: cc. N/50 NH <sub>3</sub> , 3.69, 3.75. N 1.23, 1.23. N:P::1:1.26.
Sample B.	0.0653, 0.0649 gm. substance: cc. N/20 NaOH, 39.41, 38.91. P 3.34, 3.32. 0.0980, 0.0992, 0.0990 gm. substance: cc. N/50 NH <sub>3</sub> , 4.75, 4.78, 4.69. N 1.35, 1.35, 1.33. N:P::1:1.12.

This first step in the purification of the cephalin fraction using Levene's<sup>3</sup> first method of purification splits this fraction in three different lots. The most insoluble material according to the analytical data consists in a higher percentage of the cephalin fragments that have lost their aminoethanol grouping.

These two samples of cephalin so called were joined together and submitted to the second method of purification as outlined by Levene.<sup>3</sup>



14.8 gm. were dissolved in 50 cc. of warm ether and 75 cc. of warm alcohol added. The warm solution was then precipitated with 100 cc. of a saturated alcoholic solution of cadmium chloride. A dark varnish-like precipitate (3) was formed. The dark brownish solution was decanted and studied for its cephalin content.

The solution was concentrated to dryness and the residue dissolved in ether. The filtered ether solution was evaporated and the residue emulsified with 50 cc. of water, and precipitated in the cold by 25 cc. of acetone. The precipitate was again precipitated from a minimum quantity of ether with 50 cc. of acetone. The light brown, amorphous precipitate was centrifuged off and dried to constant weight *in vacuo*. This formed a light brown powder. It weighed 0.4542 gm.

*Analyses.* 0.0691, 0.0692 gm. substance: cc. N/20 NaOH, 46.89, 46.53.  
P 3.74, 3.72.  
0.0986, 0.0978 gm. substance: cc. N/50 NH<sub>3</sub>, 3.22, 3.06.  
N 0.92, 0.88.  
N:P::1:1.87.

This analysis indicates that the purification of this fraction resulted in a fraction containing larger quantities of these fragments of cephalin which had lost their aminoethanol grouping.

*Examination of Precipitate (3).*—According to Levene this precipitate from the brain contains lecithin cadmium chloride (4) and cephalin (5) of a lower carbon content. The cephalin was extracted by two 100 cc. portions of glacial acetic acid. This glacial acetic acid solution was concentrated to dryness, emulsified with 100 cc. of water, cooled, and precipitated with 75 cc. of acetone. The precipitate was centrifuged off, dissolved in 35 cc. of ether, and precipitated in the cold with 125 cc. of alcohol. This precipitate was dissolved in 30 cc. of ether and precipitated again with 125 cc. of acetone. The brown amorphous material was centrifuged off and dried *in vacuo*. Weight 2.991 gm.

*Analyses.* 0.0978, 0.0982 gm. substance: cc. N/50 NH<sub>3</sub>, 4.96, 5.29.  
N 1.42, 1.51.  
0.0679, 0.0674 gm. substance: cc. N/20 [NaOH, 45.52, 45.13.  
P 3.71, 3.70.  
N:P::1:1.14.

2.1668 gm. of this material were hydrolyzed by boiling for 12 hours with 100 cc. of 10 per cent hydrochloric acid. The fatty acids recovered from the ether extract weighed 1.280 gm. (59.1 per cent) and had an iodine number of 109.

The aqueous hydrolysis products were treated in the usual manner. These were made up to a volume of 50 cc. Analysis for total nitrogen and by the micro Van Slyke method showed that 70.2 per cent of the total nitrogen was in the amino form.

*Analyses.* 2.00, 2.00 cc. samples: cc.  $N/50$   $NH_3$ , 2.95, 2.83.  
 Total N 0.000827, 0.000793 gm., average 0.000810.  
 2.00, 2.00 cc. samples: cc. N at 28°C. and 734 mm. pressure, 1.19, 1.21.  
 Blanks: cc. N at 28°C. and 734 mm. pressure, average 0.12.  
 Average  $NH_2$  N from 2.00 cc., 1.08 cc., 0.000569 gm.  

$$\frac{0.000569}{0.000810} = 70.2 \text{ per cent of the total nitrogen in the amino form.}$$

*Examination of Glacial Acetic Acid-Insoluble Material (4).—*This material was washed thrice with 100 cc. portions of acetic acid, thrice with 75 cc. of alcohol, and twice with 75 cc. of acetone. This was a dark brown granular material. It was dried to constant weight *in vacuo*. Weight 5.57 gm.

*Analyses.* 0.0954 gm. substance: cc.  $N/50$   $NH_3$ , 4.76.  
 N 1.40.  
 0.0690, 0.0693 gm. substance: cc.  $N/20$  NaOH, 43.52, 43.77.  
 P 3.49, 3.49.  
 N:P::1:1.13.

2.6617 gm. of this material were hydrolyzed by boiling for 11 hours with 100 cc. of 10 per cent hydrochloric acid. The fatty acids from the ether extract weighed 1.390 gm. (52.2 per cent) and had an iodine number of 111. The aqueous hydrolysis liquids were treated in the usual manner, the final volume being 50 cc. Analysis showed that 74.1 per cent of the total nitrogen was in the amino form.

*Analyses.* 2.00, 2.00 cc. samples: cc.  $N/50$   $NH_3$ , 3.87, 3.86.  
 Total N 0.00108, 0.00108 gm.  
 2.00, 2.00 cc. samples: cc. N at 28°C. and 734 mm. pressure, 1.64, 1.64.

Blanks: cc. N at 28°C. and 734 mm. pressure, average, 0.12.

Average  $\text{NH}_3$  N from 2.00 cc., 1.64 cc., 0.00080 gm.

$\frac{0.00080}{0.00108} = 74.1$  per cent of the total nitrogen in the amino form.

#### CONCLUSION.

Systematic fractionation and analyses of the cephalin fraction from corpus luteum indicate that it is similar to the same fraction from the heart, liver, and brains inasmuch as it is composed of little lecithin, cephalin, along with fragments of these lipoids.

## SYNTHESIS OF AMINO ACIDS IN THE ANIMAL BODY.

### IV. SYNTHESIS OF HISTIDINE.\*

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(Received for publication, August 27, 1926.)

The work of Ackroyd and Hopkins (1) leads to the conclusion that a diet must contain either arginine or histidine; they are, as the authors term them, "interchangeable" amino acids. This view receives support from the work of Geiling (2). The complete removal of histidine from a diet can be accomplished without much difficulty, but, with the present methods at our disposal, there is no equal assurance that arginine can be completely removed. This makes it a difficult, if not impossible matter, at present, to study the arginine problem with any definite assurance of success. The recent work of Vickery and Leavenworth (3) holds out hope that this shortcoming may soon be overcome. However, the question, Is histidine an essential amino acid? can be answered with definiteness, as Rose and Cox (4) have shown, and, as we are able to confirm. The absence of histidine from a diet produces marked loss of weight and general decline; and this irrespective of the amount of arginine which may be present.

Stewart (5) has pointed out that on a diet deficient in histidine and arginine, young rats lose weight and their allantoin excretion is markedly decreased. The addition of histidine to the deficient diet causes a resumption of growth and an increased allantoin excretion, suggesting, as Ackroyd and Hopkins had pointed out, that histidine functions as a precursor of purines in the

\* This communication was presented in abstract form before the Federation of American Societies for Experimental Biology at Cleveland, Ohio, December, 1925. See Novello, N. J., Harrow, B., and Sherwin, C. P., *J. Biol. Chem.*, 1926, lxxvii, p. liv.

animal body. The addition of arginine to the deficient diet did *not* give rise to resumption of growth nor to any increased allantoin excretion.

Obviously, histidine is an "essential" amino acid; and furthermore, histidine and arginine are *not* interchangeable. The latter statement receives added support from previous work by one of us (6). The statement was then made that "the ingestion of amino acids other than arginine [by a fowl along with benzoic acid] does not seem to increase greatly the output of ornithuric acid, apparently contradictory to the theory that histidine is convertible quite easily into arginine, for ingested histidine does not augment ornithuric acid while arginine does." The synthesis of ornithine by the hen would indicate the possible synthesis of arginine in the mammalian body and might, indeed, lead to the conclusion that arginine is not to be classed as an "essential" amino acid at all.

If we must regard histidine as an essential amino acid, we are still interested in the question, To what extent may some of the probable metabolic products of histidine replace this amino acid? Is it, indeed, possible to replace what has hitherto been regarded as an essential amino acid by some other compound? With the view that light may also be thrown on the possible catabolism of histidine in the animal body—whether the changes take place by way of the  $\alpha$ -hydroxy acid, the  $\alpha$ -keto acid, or the  $\alpha,\beta$  unsaturated compound (acrylic acid)—we selected *d,l*-imidazol lactic acid, imidazol pyruvic acid, and imidazol acrylic acid. We also tried imidazol itself.<sup>1</sup>

<sup>1</sup> The imidazol used in these experiments was purchased from Kahlbaum, and the amino acids were obtained from American firms, but they were thoroughly tested before use. The imidazol derivatives were prepared by us (results unpublished).

In the June number of the *Journal* (1926, lxxviii, 781) Cox and Rose report experiments of much the same character as ours and come to much the same conclusion. Their statement (p. 796) that, "The above experiments constitute the first successful attempt by means of growth studies to replace an 'indispensable' amino acid by any other compound whatsoever" is contrary to the facts, since a summary of our work had already been published. We reported the results of our experiments simultaneously with Cox and Rose (Cox, G. J., and Rose W. C., *J. Biol. Chem.*, 1926, lxxvii, p. iii).

## EXPERIMENTAL.

The experiments were performed on young rats. The methods followed were essentially those described by Rose and Cox (4). The source of nitrogen for the preliminary tests was unhydrolyzed

TABLE I.

*Diets of Unhydrolyzed Casein and Completely Hydrolyzed Casein.*

Composition.	Diet No.			
	100	101	102	103
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Unhydrolyzed casein.....	14.7	14.7		
Completely hydrolyzed casein....			14.2	14.05
Cystine.....	0.3	0.3	0.3	0.3
Tyrosine.....			0.3	0.45
Tryptophane.....			0.2	0.2
Dextrin.....	34.0	40.0	40.0	40.0
Sucrose.....	15.0	15.0	15.0	15.0
Lard.....	25.0	19.0	19.0	19.0
Salt mixture.....	4.0	4.0	4.0	4.0
Agar.....	2.0	2.0	2.0	2.0
Cod liver oil.....	5.0	5.0	5.0	5.0
Yeast.....				

TABLE II.

*Diet of Completely Hydrolyzed Casein from Which the Histidine and Arginine Had Been Removed.*

Composition.	Diet 201.
	<i>per cent</i>
Completely hydrolyzed casein minus histidine and arginine.....	14.05
Cystine.....	0.3
Tyrosine.....	0.45
Tryptophane.....	0.20
Dextrin.....	40.0
Sucrose.....	15.0
Lard.....	19.0
Salt mixture.....	4.0
Agar.....	2.0
Cod liver oil.....	5.0
Yeast.....	

casein and completely hydrolyzed casein. Subsequently, in the test experiments, a hydrolyzed casein from which the histidine and arginine had been removed, was used. To this mixture of unsatisfactory amino acids, definite quantities of the imidazol compounds, including histidine, were added. The diets are given in Tables I and II.

TABLE III.

*Food Consumption and Body Weight Changes on Unhydrolyzed Casein.*

Rat No.	Weight at commencement of experiment.	Days.	Diet No.	Average daily change in body weight.	Average daily food consumption.
	gm.			gm.	gm.
A	83	1-30	100	+1.12	3.6
		31-60	101	+1.42	4.8
		61-90	101	+0.96	5.0
		91-120	101	+0.79	5.9
		121-150	101	+0.55	6.6
B	79	1-18	100	+1.44	4.6
		19-36	100	+1.09	3.3
		37-60	101	+1.50	4.7
		61-90	101	+0.92	5.9
		91-120	101	+0.90	7.0
		121-150	101	+0.80	7.0
C	68	1-28	100	+1.0	3.6
		29-60	101	+1.39	4.7
		61-90	101	+0.96	5.2
		91-120	101	+0.80	6.0
		121-150	101	+0.70	8.0
D	73	1-28	100	+1.13	3.3
		29-61	101	+1.52	5.0
		62-90	101	+1.01	5.5
		91-120	101	+0.80	6.5
		121-150	101	+0.70	7.75

Preliminary experiments showed that 30 mg. of commercial yeast—as a source of vitamin B—per rat per day were sufficient for normal growth. The fat-soluble vitamin—the vitamin A and the antirachitic substance—was supplied by the addition of cod liver oil (5 per cent) to the diet. The nitrogen components of the diet made up about 15 per cent.

In Table III are summarized the results with unhydrolyzed casein. These results indicate quite clearly normal growth development in Rats A, B, C, and D. Rats X, Y, and Z were placed on completely hydrolyzed casein and the results are recorded in Table IV. While the rate of growth in the earlier stages is not so marked as when unhydrolyzed casein is used, nevertheless the

TABLE IV.  
*Food Consumption and Body Weight Changes on Diet of Completely Hydrolyzed Casein.*

Rat No.	Weight at commencement of experiment.	Days.	Diet No.	Average daily change in body weight.	Average daily food consumption.
	gm.			gm.	gm.
X	81	1-30	100	+1.16	5.0
		31-50	102	+0.51	4.2
		51-65	103	+0.60	5.0
		66-90	103	+0.80	5.7
		91-120	103	+0.60	6.2
		121-150	103	+0.91	8.1
Y	77	1-28	100	+0.98	4.6
		29-45	102	+0.33	5.2
		46-66	103	+0.61	5.6
		67-93	103	+0.69	6.0
		94-120	103	+0.77	6.6
		121-150	103	+0.79	8.0
Z	73	1-30	100	+1.02	4.4
		31-50	102	+0.47	4.5
		51-70	103	+0.39	4.8
		71-90	103	+0.80	5.9
		91-120	103	+0.86	6.6
		121-150	103	+0.81	8.3

results are sufficiently conclusive to substantiate Abderhalden's contention (7) that life can be supported where the sole source of nitrogen is the amino acids obtained by the hydrolysis of a protein. In this respect, our results are in entire agreement with those of Rose and Cox (4).

In the attempt to substitute for an essential amino acid such as histidine a compound closely related to it, we first selected imid-



TABLE V.

*Food Consumption and Body Weight Changes when Imidazol Lactic Acid (A) Is Used in Place of Histidine.*

Rat No.	Weight at commencement of experiment.	Diet No.	Days.	Average daily change in body weight.	Weight at end of each period.	Average daily food consumption.
	gm.			gm.	gm.	gm.
21	76	101	1-10	+2.8	104	6.8
		201	11-33	-0.73	88	2.3
		(201 + 0.2 per cent A.)	34-50	+1.25	109	6.0
		201	51-66	-0.60	100	2.7
		(201 + 0.4 per cent A.)	67-90	+1.26	129	6.3
		201	91-120	-0.77	106	2.4
		(201 + 0.2 per cent histidine.)	121-150	+1.45	135	7.8
12	68	101	1-10	+3.33	101.3	7.0
		201	11-30	-1.15	78.3	2.5
		(201 + 0.1 per cent A.)	31-50	+0.88	96.0	4.8
		201	51-70	-0.75	81	2.3
		(201 + 0.3 per cent A.)	71-85	+2.07	112	6.7
		201	86-100	-0.77	97	2.2
		(201 + 0.1 per cent histidine.)	101-130	+0.70	119	7.4

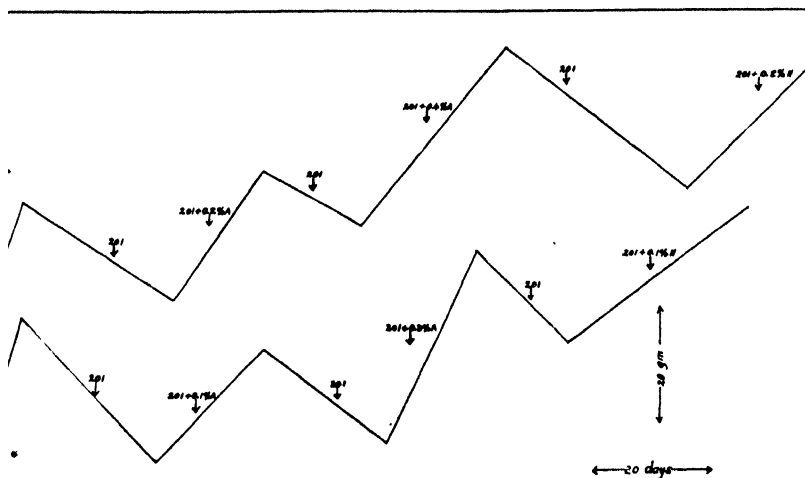


CHART I.

101 = Diet 101.  
201 = Diet 201.

A = imidazol lactic acid.  
H = histidine.

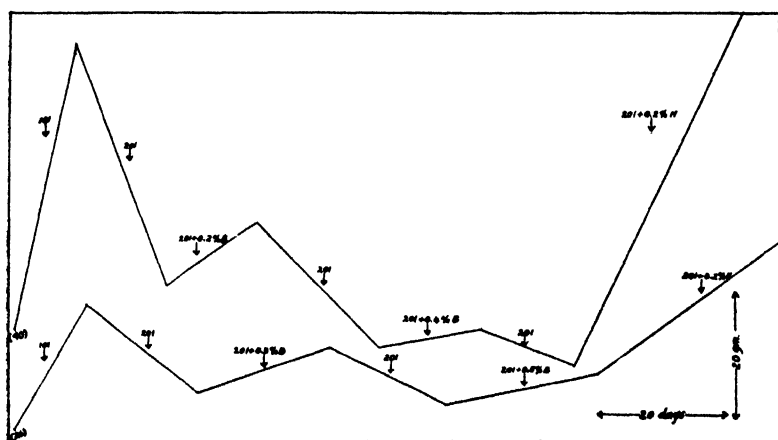


CHART II.

101 = Diet 101.  
201 = Diet 201.

B = imidazol pyruvic acid.  
H = histidine.

TABLE VI.

*Food Consumption and Body Weight Changes when Imidazol Pyruvic Acid (B) Is Used in Place of Histidine.*

Rat No.	Weight at commencement of experiment.	Diet No.	Days.	Average daily change in body weight.	Weight at end of each period.	Average daily food consumption.
	gm.			gm.	gm.	gm.
26	70	101	1-12	+1.7	90	4.4
		201	13-30	-0.8	76	2.0
		(201 + 0.3 per cent B.)	31-52	+0.3	83	3.3
		201	53-71	-0.5	74	2.1
		(201 + 0.5 per cent B.)	72-96	+0.3	79	3.4
		(201 + 0.2 per cent histidine.)	97-126	+0.75	101	5.0
40	82	101	1-10	+3.8	120	7.7
		201	11-26	-2.45	81	2.2
		(201 + 0.2 per cent B.)	27-40	+0.7	91	4.1
		201	41-60	-0.7	78	2.1
		(201 + 0.4 per cent B.)	61-77	+0.5	86	4.4
		201	78-92	-0.45	80	2.0
		(201 + 0.2 per cent histidine.)	93-120	+1.6	125	6.6

azol lactic acid. The rat was first placed upon a diet the nitrogen components of which consisted of unhydrolyzed casein (No. 101), Diet 101 being next replaced by completely hydro-

TABLE VII.

*Food Consumption and Body Weight Changes when Imidazol Acrylic Acid (C) Is Used in Place of Histidine.*

Rat No.	Weight at commencement of experiment.	Diet No.	Days.	Average daily change in body weight.	Weight at end of each period.	Average daily food consumption.
	gm.			gm.	gm.	gm.
31	82	101	1-12	+2.6	113	7.1
		201	13-30	-0.7	101	2.2
		(201 + 0.3 per cent C.)	31-45	-0.07	100	3.5
		201	46-56	-1.0	90	2.0
		(201 + 0.5 per cent C.)	57-70	-0.1	88	3.3
96	77	101	1-15	+3.1	123.5	7.1
		201	16-28	-0.9	112	2.4
		(201 + 0.5 per cent C.)	29-50	-0.1	110	3.3
		(201 + 0.2 per cent histidine.)	51-62	+3.5	152	8.5
		(201 + 1 per cent C.)	63-80	-0.15	149	2.9

lyzed casein from which the histidine and arginine had been removed (Diet 201), and the imidazol lactic acid added to Diet 201 after the latter diet had shown unmistakable evidence of insufficiency. The results are given in Table V (see also Chart I).

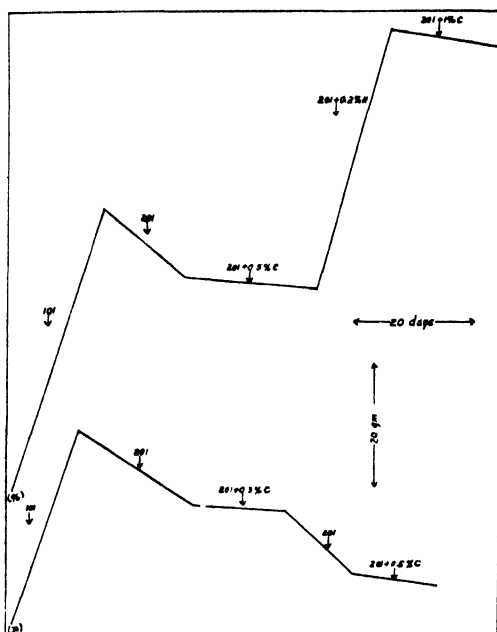


CHART III.

101 = Diet 101.

C = imidazol acrylic acid.

201 = Diet 201.

H = histidine.

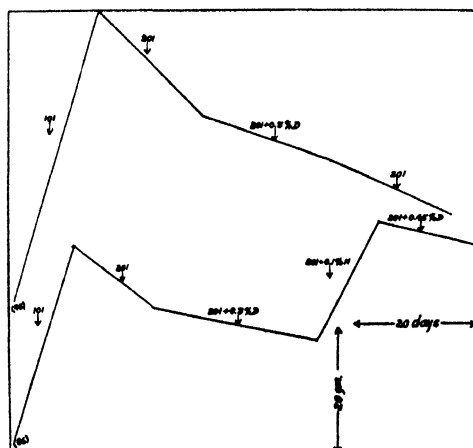


CHART IV.

101 = Diet 101.

D = imidazol.

201 = Diet 201.

H = histidine.

The duplicate results show two things: first, that a diet from which all traces of histidine have been removed (and as much of the arginine as present methods make possible), and which is therefore a deficient diet (Diet 201), may be markedly improved by the addition of histidine, confirming, thereby, the essential

TABLE VIII.

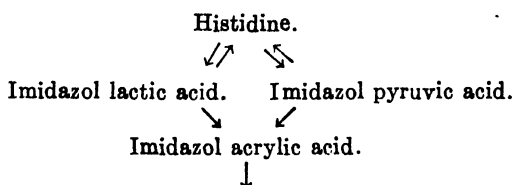
*Food Consumption and Body Weight Changes when Imidazol (D) Is Used in Place of Histidine.*

Rat No.	Weight at commencement of experiment.	Diet No.	Days.	Average daily change in body weight.	Weight at end of each period.	Average daily food consumption.
	<i>gm.</i>			<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
93	86	101	1-14	+3.3	133	7.8
		201	15-31	-1.1	116	2.5
		(201 + 0.3 per cent D)	32-52	-0.3	109	3.6
		201	53-70	-0.5	100	2.8
66	72	101	1-10	+3.1	103	7.7
		201	11-23	-0.75	93	2.0
		(201 + 0.3 per cent D.)	24-50	-0.2	88	3.2
		(201 + 0.1 per cent histi- dine)	51-60	+2.0	107	8.2
		(201 + 0.45 per cent D.)	61-76	-0.25	103	3.3

nature of this amino acid; secondly—and this is the more striking because the more novel result—imidazol lactic acid, a compound closely related, chemically, to histidine and a possible intermediary compound in the metabolism of histidine in the body, serves as a very good substitute for histidine in the diet.

Somewhat less efficient than imidazol lactic acid, but nevertheless showing distinct beneficial effects, is imidazol pyruvic acid, the results with which are given in Table VI (see also Chart II). Less efficient than the imidazol pyruvic acid, but still showing slight beneficial effects, is imidazol acrylic acid (Table VII and Chart III). Imidazol itself has no value when used with a histidine-free diet (Table VIII and Chart IV).

From the results obtained, one may picture the probable metabolism of histidine in the body as follows:



These experiments indicate the possibility of the synthesis of histidine from imidazol lactic acid and imidazol pyruvic acid. They also tend to show that either the  $\alpha$ -keto acid or the  $\alpha$ -hydroxy acid may be the first step in the catabolism of histidine, and that the reaction is reversible. If this view be accepted, then from our experiments it seems probable that the  $\alpha$ -hydroxy acid is more easily formed than the  $\alpha$ -keto acid, which is contrary to what is generally accepted. The  $\alpha$ - $\beta$  unsaturated acid (the acrylic acid) may be one of the intermediary steps in histidine catabolism, since feeding experiments have shown that it is completely oxidized in the animal body. It represents, in any case, a secondary reaction in the catabolism of histidine, since the acrylic acid cannot be resynthesized into histidine.

#### SUMMARY.

1. The importance of histidine in the diet is conclusively demonstrated.
2. Imidazol pyruvic acid is able, to some extent, to replace histidine in the diet; but imidazol lactic acid is much more efficient in this capacity, while imidazol acrylic acid is less so.
3. Imidazol alone has no value when used with a histidine-free diet.

4. The probable metabolism of histidine in the body is discussed.

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## BASIC PROTEINS.

### I. THE NITROGEN DISTRIBUTION AND THE PERCENTAGES OF SOME AMINO ACIDS IN THE PROTAMINE OF THE SARDINE, *SARDINIA CÆRULEA*.\*

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Protamines were discovered in 1874 by Miescher (1) in the spermatazoa of the salmon. Subsequently it has been shown that protamines are present in the spermatazoa of the carp (2), the common herring (2-8), two species of mackerel (9, 10), three of sturgeon (3, 11-15), four of salmon (2-5, 11, 16-21), and in eight less common species of fishes (22-25). Protamines have not been found in plants nor in animals other than in the spermatazoa of fishes.

Our knowledge of the protamines is chiefly due to Kossel and his coworkers. Their investigations have shown that the protamines are unique as compared to other proteins, particularly in respect to their amino acid composition. They are the most basic of the proteins. In the majority of cases, their basicity is due to an extremely high content of arginine. With a few exceptions protamines have been found to contain from about 58 to 92 per cent of this amino acid. Lysine, the only amino acid besides arginine found to an unusual extent in a protamine, constitutes 28.8 per cent of  $\alpha$ -cyprinine, a protamine of the carp (2).

Protamines are further characterized by the presence of relatively few amino acids. In no case has it been proved that a protamine contains more than five different amino acids (26).

\* The writer is indebted to Mr. C. B. Andrews of the California State Bureau of Fisheries, Terminal Island, California, for assistance in the collection of the sardine testicles and to Mr. G. L. Samuelson for the preparation of the protamine sulfate used in these experiments.

Because of their high proportion of arginine and low content of other amino acids Kossel has advanced the assumption that the protamines are the simplest proteins. Objections to this conception have been raised by Abderhalden (18, 27) who believes that the protamines may be just as complicated as other proteins.

Although the protamines of numerous species of fishes have been studied, in only a few cases (28, 29) has quantitative information concerning their amino acid composition been obtained. For this reason and because of the obvious importance of the protamines to the problems of heredity and to the general reactions of cells, the present studies were undertaken.

#### EXPERIMENTAL.

Testicles of the sardine, *Sardinia caerulea*, were obtained from the fish canneries at East San Pedro, California, during the spawning season in May. The ovaries and testicles can readily be distinguished as the former are yellowish, granular, and covered with a network of fine blood vessels while the latter are smooth, grayish, flat, lobular organs adjacent to the kidneys and the ventral surface of the fish. The testicles are about 10 cm. in length in a sardine three times as long.

The testicles were obtained from sardines caught in the Pacific ocean about 12 hours previously. They were brought directly to the laboratory, placed in a cheese-cloth bag, and allowed to drain for several hours in the ice box. The protamine was extracted from the finely ground testicles with 1 per cent sulfuric acid. The protamine sulfate was precipitated with alcohol and purified according to the procedure of Kossel (3, 11, 23). The white, flocculent precipitate was thoroughly desiccated with alcohol and ether, and dried *in vacuo* over sulfuric acid. From 3015 gm. of the moist testicles 15 gm. of white, powdery protamine sulfate were obtained. This product contained 18.35 per cent of nitrogen (calculated on an ash- and moisture-free basis).

The distribution of nitrogen and the percentages of some amino acids of the sardine protamine were determined by the Van Slyke method and the colorimetric procedure of Folin and Looney (30). The results of these analyses are given in Tables I, II, III, and IV.

TABLE I.  
*Distribution of Nitrogen in Four Groups.\**

Form of nitrogen.	Sardine protamine.		
	I	II	Average.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Humin N.....	0.51	0.52	0.51
Amide N.....	0.15	0.16	0.15
Basic N.....	10.26	10.47	10.36
Non-basic N.....	7.35	7.29	7.32
Total.....	18.27	18.44	18.34

\* Results expressed in percentages of the ash- and moisture-free protamine. Nitrogen content of the protamine, 18.35 per cent.

TABLE II.  
*Distribution of Nitrogen in the Protamine of the Sardine as Determined by the Van Slyke Method.\**

Form of nitrogen.	I	II	I	II	Average.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Amide N.....	0.0037	0.0038	0.85	0.87	0.86
Humin N.....	0.0122	0.0125	2.79	2.86	2.83
Arginine N.....	0.1208	0.1224	27.65	28.02	27.83
Cystine N.....	0.0033	0.0020	0.75	0.46	0.60
Histidine N.....	0.1006	0.1002	23.03	23.01	23.02
Lysine N.....	0.0216	0.0263	4.94	6.02	5.48
Amino N of filtrate.....	0.1158	0.1104	26.51	25.27	25.89
Non-amino N of filtrate.....	0.0606	0.0646	13.87	14.79	14.33
Total N regained.....	0.4386	0.4422	100.39	101.30	100.84

\* Sample I, ash- and moisture-free, 2.39 gm. protamine; 0.4368 gm. nitrogen. Sample II, ash- and moisture-free, 2.39 gm. protamine; 0.4368 gm. nitrogen.

A sample of about 6 gm. of the air-dried protamine sulfate was hydrolyzed for 26 hours with 250 cc. of 20 per cent hydrochloric acid. Equal aliquots were taken for analysis.

The total nitrogen regained was corrected for solubility of the bases. The phosphotungstate precipitate was decomposed by the amyl alcohol-ether method.

TABLE III.  
Percentages of Some Amino Acids in Sardine Protamine.\*

Amino acid.	I	II	Average.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Arginine .....	15.70	15.91	15.80
Cystine.....	1.18	0.72	0.95
Histidine .....	15.73	15.67	15.70
Lysine.....	4.71	5.74	5.22
Tyrosine .....	1.09		1.09
Tryptophane.....	0.87		0.87

\* Percentages based on the ash- and moisture-free protamine. Arginine, cystine, histidine, and lysine determined by the Van Slyke method. Tyrosine and tryptophane determined colorimetrically by the method of Folin and Looney (30). For this purpose a separate 1.0 gm. sample of sardine protamine sulfate was hydrolyzed with barium hydroxide for 48 hours.

TABLE IV.  
Free Amino Nitrogen of the Sardine Protamine Compared with the Lysine Nitrogen.\*

N gas from 5 cc.	Pressure.	Temperature.	Amino N in 100 cc.	Ratio of amino N to total N.	One-half lysine N by Van Slyke method.
<i>cc.</i>	<i>mm.</i>	<i>°C.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
0.605	750.5	20.5	0.0068	9.98	2.74

\* 0.3734 gm. of the ash- and moisture-free protamine was dissolved in water and diluted to 100 cc. 5 cc. of this solution were used for determination of the amino nitrogen in the Van Slyke micro amino nitrogen apparatus. The total nitrogen in the 100 cc. of solution was 0.0681 gm.

#### DISCUSSION.

No analysis of the protamine from the sardine has previously been reported. The protamine from only one other member, *Clupea harengus*, of the herring (Clupeidæ) family has been studied.

The figures for the distribution of amino acids in the protamine of the sardine represent maximal values since they were obtained by the Van Slyke and colorimetric procedures.

The data indicate that the protamine of the sardine, *Sardinia cærulea*, differs from that of the common herring, *Clupea harengus*. The former protamine is found to contain 15.80 per cent of arginine, 15.70 per cent of histidine, 5.22 per cent of lysine, and

smaller percentages of tyrosine, cystine, tryptophane, and ammonia. With the exception of arginine these amino acids are lacking in the herring protamine. Furthermore the latter contains 82.2 per cent of arginine. Two other protamines with a low arginine content have been studied. The  $\alpha$ -cyprinine of carp contains 4.9 per cent of arginine and the crenilabrine from *Crenilabrus pavo* 23.7 per cent. The protamine from the sturgeon, *Accipenser sturio*, resembles that of the sardine in having histidine and lysine as well as arginine.

Three other protamines besides the sardine have been found to contain tyrosine (2, 22, 23) and one other tryptophane (26). The low percentage of amide ammonia obtained from the acid hydrolysate of the sardine protamine indicates the presence of small amounts of the dicarboxylic amino acids, the decomposition of amino acids, or the presence of amino acid anhydrides (cyclic diacipiperazines) from which ammonia is believed to be derived (31). The presence of cystine in the sardine protamine seems certain since the blue color distinctly obtained with Folin's phenol reagent in the acid hydrolysate of the protamine is specific for cystine (in the reduced form, cysteine).

An inspection of Table IV shows that the free amino nitrogen, obtained by the reaction of nitrous acid with the native protamine, is considerably in excess of one-half of the lysine nitrogen. These values should be equivalent if, according to a current theory, no amino groups other than that on the epsilon carbon of lysine are free in the protamine molecule. A similar disagreement has been reported for another protamine. According to Kossel and Dakin (2) one-half of the lysine nitrogen from the  $\alpha$ -cyprinine of the carp is 15.2 per cent of the total nitrogen while Kossel and Cameron (32) found that the free amino nitrogen of this protamine was 23.6 per cent. Carp protamine was found to contain 19.5 per cent of free amino nitrogen by the present writer (unpublished results).

It appears, from the results obtained, that six different amino acids account for about 40 per cent of the amino acids present in the sardine protamine. Approximately 55 per cent of the total nitrogen is found in the basic amino acids but the latter constitute only about 37 per cent of the protamine. Thus it would seem that other monoamino acids, left undetermined by the

methods employed, are present. The evidence obtained is not in support of the view that the sardine protamine is of simple construction. Kossel's assumption that the protamines are the simplest proteins may be true in some cases but it appears unlikely for the sardine,  $\alpha$ -cyprinine of the carp, and for all other species not possessing a high proportion of a single amino acid.

#### SUMMARY.

1. The protamine sulfate from the ripe testicles of the sardine, *Sardinia caerulea*, has been purified and obtained as a white powder. Analyzed by the Van Slyke method, the basic amino acids of the protamine were found to comprise less than one-half of the total amino acid content.

2. Data obtained by the Van Slyke method and the Folin and Looney colorimetric procedure indicate that small percentages of ammonia, tyrosine, cystine, and tryptophane are present in the sardine protamine.

3. The free amino nitrogen obtained from the sardine protamine was in excess of one-half of the lysine nitrogen. This disagreement suggests the presence of free amino groups other than that on the terminal carbon of lysine. A similar conclusion is drawn from the data reported for a protamine of the carp.

4. No evidence is obtained in support of the theory that the protamines are the simplest proteins.

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# THE DETERMINATION OF HYDROGEN IONS IN THE BLOOD WITH THE AID OF THE DUBOSQ COLORIMETER AND ORTHO-CHROM-T OR PARA-NITROPHENOL.

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Since a large variety of apparatus makes a laboratory very crowded, an attempt has been made to make a number of quantitative determinations, such as might be done in a hospital laboratory, with the same apparatus. For a number of years we have made considerable use of the Dubosq colorimeter, and the present paper is an adaptation of it to the determination of hydrogen ions in blood plasma. For this work it is necessary to have an indicator that shows change only in intensity of color with degree of dissociation and not a mixture of two colors such as is the case with phenol red. There are very few such indicators with the sensitive portion of their range within the physiological limits of blood pH. Of these, cyanine (quinoline blue) is very strongly affected by proteins or some other constituent of plasma so that we have never been able to obtain a color match. Para-nitrophenol is rather a strong precipitant of the plasma proteins but we have been able to use it.

## *Ortho-Chrom-T.*

The best indicator that we have found is ortho-chrom-T, which we obtained from the Farbwerke vorm. Meister Lucius und Brüning, Hoechst. This indicator, which is used as a photosensitizer, is easily oxidized by atmospheric air, but we did not find any destruction of the indicator during the short period necessary to make the determinations. 0.1 gm. is dissolved in 10 cc. of alcohol and this is mixed with 90 cc. of distilled water and kept in a bottle with a dropping pipette. Two precautions have to be taken

in the determination. One is to guard against loss of  $\text{CO}_2$  from the blood plasma, and the other is to secure absolute symmetry in the optical arrangements on the two sides of the colorimeter. We have used mainly the "biological colorimeter" as modified slightly for us by the Bausch and Lomb Optical Company, and with a few changes made by a mechanic. These modifications do not interfere with its use for other work.

In order to prevent loss of  $\text{CO}_2$  from the diluted blood plasma, two methods have been tried. Perhaps the best is the use of the cup shown in Fig. 1 which has a fused-on glass bottom and a removable cover, which, when seated, gives a depth of fluid of exactly 20 mm. Indicator in distilled water is poured into the cup and the plasma is introduced through a long fine point of a pipette into the bottom of the cup, causing the indicator solution to overflow. The cover is then quickly seated without air bubbles, and the cup grasped with a towel and rotated violently to stir up the contents. Tight fitting of the glass cover prevents the loss of  $\text{CO}_2$ .

Another method is the use of the cup with side neck shown in Fig. 2. This cup has the top and bottom fused on, the distance between them being exactly 20 mm., and has a side neck for filling. 4.5 cc. of distilled water containing the indicator are introduced, the top level being up in the side neck and a layer of paraffin oil floated over it. The plasma is introduced by inserting the finely drawn out tip of a pipette down the side neck; after introducing the plasma the contents are stirred with the pipette point or a needle.

For micro determinations, the cup with side neck shown in Fig. 3 has been used with the Buerker colorimeter; the top and bottom are fused on, being exactly 10 mm. apart. This cup was made by the Bausch and Lomb Optical Company. The original cups of the Buerker colorimeter have not the bottoms fused on and therefore they are liable to leak. Also the arrangement is awkward for introducing plasma into the bottom of the cup and getting the cover on without air bubbles.

The arrangement of the cup with side neck in the biological colorimeter is shown in Fig. 4. The cup with side neck is placed below the plunger cup on the left hand side, the plunger cup being filled with distilled water. On the right hand side a cup similar to Fig. 1 is placed below the plunger cup, this cup shown in Fig. 1

being known as the accessory cup and containing a mixture of distilled water and plasma to give the same degree of cloudiness and yellow color as the plasma on the left hand side containing



FIG. 1.



FIG. 2.

the indicator. The plunger cup on the right hand side contains indicator in 0.01 N sodium carbonate solution in which it is approximately 100 per cent dissociated.

The arrangement in Fig. 4 may be modified in order to avoid the cup with side neck by substituting for it another accessory cup as shown in Fig. 1.



FIG. 3.



FIG. 4.

The optical system is now theoretically symmetrical, but in practice is found not to be so. It seems that the indicator increases the colloidal aggregation of the proteins of the plasma and hence the left hand side shows more cloud than the right hand

side. In order to avoid this, barium hydroxide was added to the plunger cup on the right hand side and immediately precipitated by action of the sodium carbonate, forming a cloud.

*Procedure.*

The blood is drawn by means of an oiled Luer syringe and immediately transferred to a centrifuge tube previously prepared. The centrifuge tube contains enough 30 per cent potassium oxalate solution to make 0.1 per cent in the blood (for animals double quantity is used). A layer of paraffin oil is floated over the potassium oxalate solution. The needle of the syringe is inserted into the potassium oxalate solution and the blood is transferred to the centrifuge tube and immediately centrifuged. A 1 cc. pipette, graduated in 0.01 of a cc., with a very long finely drawn out tip is inserted into the plasma and the plasma sucked up into it.

Since the plasma will dilute the indicator, the indicator solution in the cup with the side neck is made stronger than that in the sodium carbonate solution used as a standard. The plunger cup on the left is filled with  $H_2O$ . In the plunger cup on the right hand side are placed 5 cc. of sodium carbonate solution plus 2 drops of 0.05 N  $Ba(OH)_2$  solution and 3 drops of the indicator. In the cup with side neck on the left hand side are placed 4.5 cc. of distilled water plus 3 drops of indicator, and oil is floated over it. Then the tip of the pipette containing the plasma is introduced and 0.5 cc. of plasma is allowed to enter, and then stirred. Since the pipette is narrow, the portion of the plasma that has come in contact with the air is not used and does not diffuse into the portion that is used. In the accessory cup on the right hand side is placed a mixture of 5 cc. of  $H_2O$  plus 0.5 cc. of plasma, and the readings are made. Since the side neck cup on the left hand side is 20 mm. deep, the readings in mm. on the right hand side divided by 0.2 would give the percentage dissociation of the indicator. This is then found on the vertical scale in Fig. 5 and the horizontal line run to the diagonal marked *O.C.T.* The point of intersection is traced vertically and the pH read off.

This method was standardized with ox serum in the Clark hydrogen electrode at 27°. If there is any change in temperature

or salt content or any variation in the indicator, it should be re-standardized by finding one point on Fig. 5 and drawing a diagonal parallel to the one given for 27°.

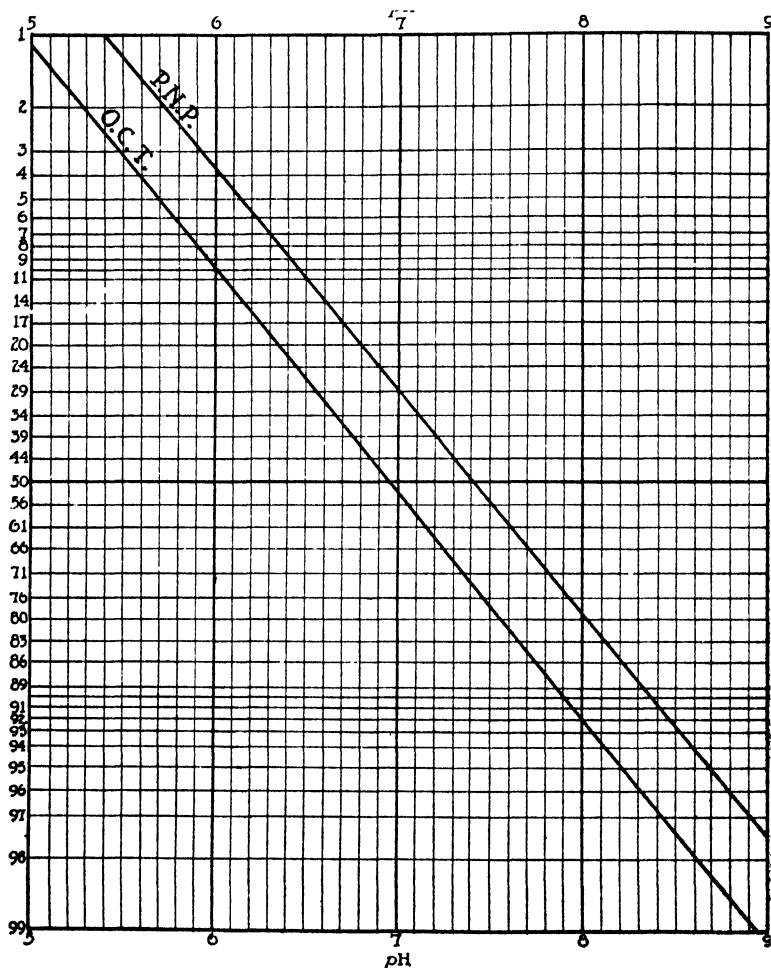


FIG. 5.

Since oil is troublesome to clean from the side neck of the cup, it is more convenient to use the cup shown in Fig. 1. This cup as made for us holds exactly 3 cc. when the cover is seated. It is filled to overflowing with a mixture of 4.5 cc. of  $H_2O$  plus 3 drops

of the indicator and 0.3 cc. of the plasma is introduced carefully *at the bottom* avoiding any mixing until the cover is seated. The cover is quickly seated and the cup is grasped in a towel and rotated until mixed, when it is placed below the plunger cup on the left hand side of the colorimeter. The rest of the procedure is the same as the above.

*Para-Nitrophenol.*

The technique with para-nitrophenol is only slightly different from the above. Since the indicator is rather pale, 3 drops of a 1 per cent solution in water were used with the same quantities of solution to which 2 drops of indicator were added in the above technique. According to Michaelis, colors can be matched better when viewed through a blue glass, but we did not find this to be any improvement. The chart in Fig. 5 was used in the same way as previously except that the diagonal marked *P.N.P* is utilized.





## THE HYDROLYSIS OF CORN-STARCH BY COMMERCIAL PANCREATIN.

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The hydrolysis of potato starch by pancreatin has been the subject of much investigation, particularly by Sherman and his pupils (1), who have studied the action of both malt and pancreatic amylase upon potato starch, especially with reference to optimum concentration of enzyme, substrate, pH value, and concentration of activating salts. The best conditions of hydrolysis were at (2) 40° in the presence of sodium chloride and disodium phosphate which acted as promoters or activators to the diastase. The favorable influence of these two salts is explained by the maintenance of a hydrogen ion concentration most favorable to the activity of the amylase. Potassium and ammonium chlorides are mentioned as exerting the same influence as the sodium chloride, while the combined effect of sodium carbonate and disodium phosphate was found to be the same as when the latter salt was used alone.

The hydrolysis of potato starch proceeds more rapidly and completely than the hydrolysis of corn-starch. This fact, which was brought to the attention of the authors by Dr. David Klein, led to the present investigation, which deals with the most favorable conditions for the hydrolysis of corn-starch.

### EXPERIMENTAL.

#### *Method.*

The method used in following the hydrolysis was to add a definite amount of pancreatin, usually dispersed in a definite amount of water, to the starch solution at 40°, from which portions were withdrawn from time to time for analysis.

The hydrolyses were always made on 98 cc. of a 2 per cent starch solution. The per cent of moisture in the starches was first determined by drying them in a vacuum oven at 60°. The amount of starch taken was such as to give a 2 per cent solution of the dried substance. The starch was placed in a 200 cc. Erlenmeyer flask, and in case salts were introduced, solutions of them were added at this point, together with an amount of water necessary to make 98 cc. of the substrate solution after the introduction of the solution of pancreatin. The contents of the flask were then brought slowly to boiling under a reflux condenser, and kept at the boiling point for 20 minutes. At the end of this time, the flask was immersed in a water thermostat regulated at 40° where it was allowed to attain the temperature of the bath.

The pancreatin was always added in solution. The desired amount of enzyme was carefully weighed, and was then introduced into a definite volume of water at 40°. If activating salts had been added to the starch, the enzyme was introduced into water containing the same concentration of activants. Aliquots were then withdrawn from the pancreatin solution and added to the starch solution. The concentration of enzyme solution was so adjusted that 10 cc. contained 5 mg. The substrate solution was always of such concentration that after the addition of the water containing the enzyme, the total volume was 98 cc. As the hydrolysis proceeded, 5 cc. portions of the substrate were pipetted off from time to time, and the maltose content (anhydrous) determined. The reducing sugars were determined volumetrically by the method of Shaffer and Hartmann (3).

#### *Materials.*

The corn-starch used in this investigation was a commercial product manufactured by the Corn Products Refining Company under the trade name of Pearl Globe (No. 144), and was furnished by courtesy of this company. The potato starch was untreated starch sold by the Central Scientific Company, Chicago. The pancreatin preparation was a commercial product of the Wilson Laboratories, Chicago, generously donated by Dr. David Klein. Its diastatic power was carefully determined and found to have a value of 66 on Sherman's new scale (4). The water used was all redistilled over alkaline permanganate and carefully protected from

the air. Salts added with the pancreatin were always of the highest purity obtainable.

### Results.

Before attempting to study the saccharogenic power of pancreatin on corn and potato starches, a preliminary microscopic study was made in order to ascertain the extent of "solution" of the boiled substrate solutions with the idea of determining if differences of dispersion would influence very greatly the saccha-

TABLE I.  
*Hydrolysis of Corn and Potato Starches by 5 Mg. of Enzyme.*

Time.	Maltose content in 5 cc. (no activants).		Maltose content in 5 cc. (activants added).	
	Potato starch.	Corn-starch.	Potato starch.	Corn-starch.
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
0.75			42.2	36.0
1.50			56.2	49.4
2.50		15.8	61.0	53.9
3.50	25.9			
5.00		29.3		
6.25	38.8			
8.00		40.4		
8.50	48.2			
10.50		44.4		
11.00	53.9			
21.00	64.5	54.4	71.2	62.3
	(64.5 per cent starch.)	(54.4 per cent starch.)	(71.2 per cent starch.)	(62.3 per cent starch.)

rogenic power of the enzyme in its action on corn and potato starches. Upon studying a boiled 2 per cent solution of potato starch microscopically, the granules were found to be greatly enlarged. A granular structure was still apparent, especially after staining with iodine. A corn-starch solution of the same concentration, and similarly treated, gave the same general appearance especially in regard to the existence of granules, although the latter did not appear to be swollen to the same extent as the potato starch granules. A 2 per cent corn-starch solution was prepared from the corn-starch after it had been rendered soluble according

to the formula of Lintner (5). When this solution was studied under the microscope, the starch granules could not be detected even after staining with iodine. Autoclaving gave similar results; the granules were disrupted upon boiling. Having been able apparently to disperse the two starches completely, a quantitative study

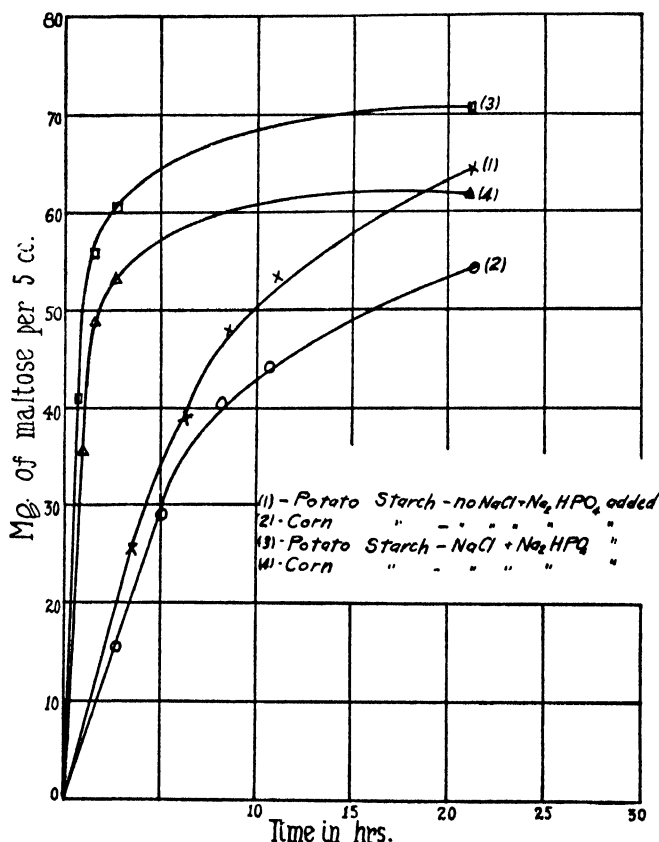


FIG. 1. Rates of hydrolysis of corn and potato starches by 5 mg. of pancreatin.

was undertaken to determine the rate of conversion of the two, as well as the effect of dispersion.

In determining the rates of conversion of corn and potato starches, 98 cc. of a 2 per cent solution of each starch were hydrolyzed by 5 mg. of pancreatin without the addition of sodium chlo-

ride and disodium phosphate. The potato starch was hydrolyzed not only more rapidly, but also more completely. If, on the other hand, 300 mg. of sodium chloride and 7 cc. of fiftieth molar disodium phosphate were added to the substrates, the rate of conversion was increased enormously; the potato starch, however, was still more readily acted upon than the corn-starch. This can be seen from the results given in Table I and Fig. 1.

The rates of conversion of the two starches were next studied when the amounts of pancreatin added were 3 and 1 mg. per 98 cc. of a 2 per cent substrate solution containing the 300 mg. of sodium

TABLE II.

*Hydrolysis of Corn and Potato Starches by Different Amounts of Pancreatin.*

Time.	Hydrolysis by 3 mg. of pancreatin (activants added).		Hydrolysis by 1 mg. of pancreatin (activants added).	
	Potato starch.	Corn-starch.	Potato starch.	Corn-starch.
hrs.	mg.*	mg.*	mg.*	mg.*
0.50	21.4	18.1	6.9	5.7
1.50	46.6	40.4		
2.00			25.9	22.6
4.50	61.0	52.7		
5.00			47.6	41.0
7.50			55.6	47.6
26.00	71.7	64.0	63.4	58.9
	(71.7 per cent of total starch.)	(64.0 per cent of total starch.)	(63.4 per cent of total starch.)	(58.9 per cent of total starch.)

\* Maltose per 5 cc. of solution.

chloride and 7 cc. of fiftieth molar disodium phosphate. Results are given in Table II and Fig. 2.

It will be seen that in each of the above cases the rate of conversion of potato starch was more rapid than the rate for the corn-starch; also that the speed increased with the concentration of pancreatin.

If after the hydrolysis of corn-starch by 1 mg. of pancreatin, 2 more mg. were added and the reaction was allowed to proceed for 20 hours more, the maltose content rose from 58 to 65 per cent of the total starch first present. An additional 2 mg. increased the yield to 67 per cent.

Having established the fact that corn-starch is converted into maltose more slowly than the same amount of potato starch, attempts were next made to increase the rate of hydrolysis of the former starch. Corn-starch was rendered soluble according to the formula of Lintner. Microscopic examination of a solution of this

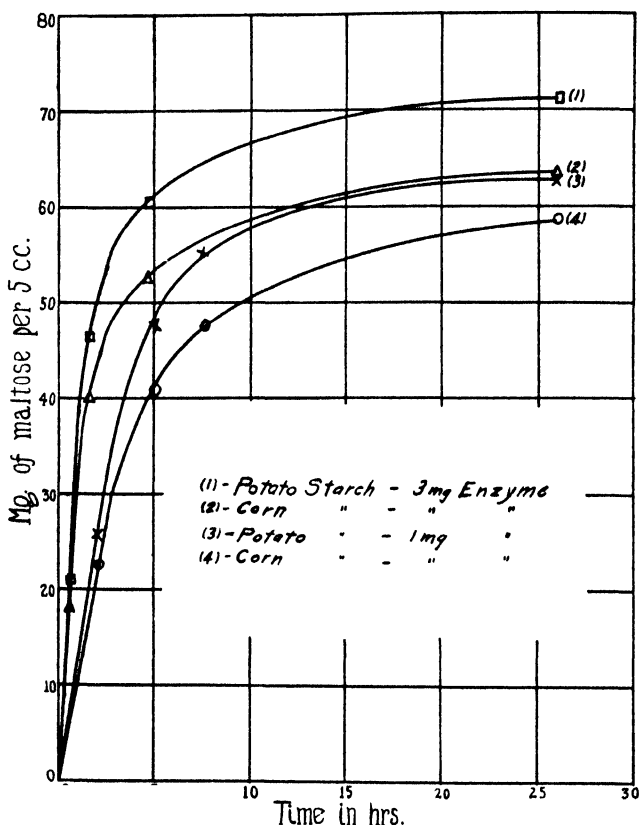


FIG. 2. Rates of hydrolysis of corn and potato starches with different amounts of pancreatin.

soluble corn-starch showed no starch granules. If this substrate was hydrolyzed more rapidly than the untreated starch, it would tend to demonstrate that the speed of conversion was dependent upon the degree of dispersion of the starch granules, provided that the material did not undergo any deep seated chemical changes during the process of rendering it soluble.

98 cc. of a 2 per cent solution of this soluble corn-starch containing sodium chloride and disodium phosphate were hydrolyzed by 5, 3, and 1 mg. of pancreatin, respectively. A blank run was always made to obtain the reducing power of the starch. In no case was the speed of conversion increased over the speed of the untreated corn-starch. Table III shows the results obtained in the hydrolysis by 5 mg. of enzyme.

The dry corn-starch was next treated with steam for 30 minutes under a pressure of 3 atmospheres. The starch so treated appeared to be partially dextrinized, but upon the hydrolysis of 98 cc. of a 2 per cent solution by 5, 3, and 1 mg. of enzyme, no increase in the rate was observed. This can be seen in Table IV in which

TABLE III.

*Effect of Starch Rendered Soluble According to Lintner on the Rate of Enzymatic Conversion.*

Time.	Untreated corn-starch. Maltose per 5 cc.	Starch rendered soluble according to Lintner. Maltose per 5 cc.
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>
0 75	36 0	37.2
1 50	49.4	49.3
2.50	53 9	53.9
21.00	62.3	64.0

NaCl + Na<sub>2</sub>HPO<sub>4</sub> added in each case.

comparisons in the maltose formation of untreated and autoclaved corn-starch by 3 mg. of enzyme are made.

The fact that the hydrolysis of the autoclaved starch proceeded more rapidly at the very first of the conversion was, no doubt, due to the fact that the starch was partially hydrolyzed into dextrans. Treating the dry starch under pressure, however, did not increase the rate of conversion. Results of identically the same order were obtained when the hydrolysis took place with both 5 and 1 mg. of enzyme.

The effect of freezing a starch solution was also tried. The 2 per cent solution of corn-starch was first boiled, after which it was frozen solidly and left for an hour. After melting it was again boiled, cooled to 40°, and the necessary amount of sodium chloride and phosphate added together with 5 mg. of pancreatin. The



results in Table V show that the freezing does not increase the rate of conversion; in fact, there is a slight decrease in speed.

A study was also made upon the hydrolysis of corn-starch which had been extracted with ether, since it was possible for the corn-starch to contain a slight amount of oil, a fact which might account for its slower enzymatic hydrolysis. From this ether-extracted starch the usual amount of a 2 per cent solution was prepared. This was then hydrolyzed with 5 mg. of enzyme after the usual

TABLE IV.

*Effect of Autoclaving Dry Corn-Starch upon the Rate of Enzymatic Hydrolysis.*

Time.	Untreated corn-starch. Maltose per 5 cc.	Autoclaved corn-starch. Maltose per 5 cc.
hrs.	mg.	mg.
0.50	18.1	21.4
1.50	40.4	39.8
4.50	52.7	52.2
26.00	64.0	64.0

NaCl + Na<sub>2</sub>HPO<sub>4</sub> added in each case.

TABLE V.

*Effect of Freezing and of Ether-Extracting Corn-Starch upon the Rate of Enzymatic Hydrolysis.*

Time.	Unaltered corn-starch. Maltose per 5 cc.	Frozen corn-starch. Maltose per 5 cc.	Ether-extracted corn-starch. Maltose per 5 cc.
hrs.	mg.	mg.	mg.
0.75	36.0	35.5	36.0
1.50	49.4	46.2	49.3
2.50	53.9	50.2	54.0
21.00	64.0	63.4	64.0

activants had been added. The results of the conversion of starch so treated are also given in Table V.

From these results it can be seen that neither freezing nor ether-extracting the corn-starch increased the rate of conversion. A solution of the starch was next autoclaved for 5 hours under a pressure of 5 atmospheres. By this treatment the substrate was made soluble. When first boiled with water, the solution was slightly cloudy, but after the treatment under pressure it became clear. No starch granules could be observed. In preparing the

solution 2 gm. of starch were boiled with 82 cc. of water, autoclaved, and 7 cc. of fiftieth molar disodium phosphate were added with 300 mg. of sodium chloride in 5 cc. of water. 1 mg. of enzyme was then added in 4 cc. of water, and 5 cc. aliquots were used for analysis from time to time. The pressure treatment converted some of the substrate into products which reduced Fehling's solution so that two blanks were always run. Table VI compares the rate of hydrolysis with that of the unautoclaved starch solution.

These results show that the rate of hydrolysis of the autoclaved solution was at first greater than the untreated substrate. This was probably due to the fact that the pressure treatment converted some of the starch into dextrans which were more readily acted upon by the enzyme. This point was substantiated by the fact

TABLE VI.

*\*Effect Which Autoclaving Corn-Starch Solution Has upon the Rate of Enzyme Hydrolysis.*

Time.	Unautoclaved corn-starch. Maltose per 5 cc.	Autoclaved solution of corn-starch. Maltose per 5 cc.
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>
0.50	5.7	9.8
2.00	22.6	25.2
5.00	41.0	34.2
7.50	47.6	
28.00	58.9	52.5

that the addition of alcohol precipitated a white substance from the autoclaved starch.

In the above case the activants were added after pressure treatment. When they were added before autoclaving the starch, the rate of hydrolysis was decreased enormously; at the end of 5 hours, 5 cc. of substrate solution contained only 12 mg. of sugar, while at the end of 26 hours the total quantity of maltose formed was only 33 per cent of the total weight of starch. In attempting to find an explanation for this result, the hydrogen ion concentration of the starch solutions was taken after hydrolysis was complete. The pH of the solution in which the sodium chloride and phosphate were added after pressure treatment was found to be 7.0 (determined by indicator method), a pH which Sherman (6) found to be most favorable for conversion with pancreatic

amylase. If, on the other hand, the activants were added before autoclaving, the solution at the completion of the action had a pH of 5.0 to 5.2, a concentration of hydrogen ions which is not very favorable to the activity of pancreatin. This difference in pH probably accounts for the large variation in the rate of conversion. If the starch solution was prepared by merely boiling the substrate with water, the pH as well as the rate of hydrolysis was identically the same whether the activants were added before or after boiling. This treatment of a starch solution did not affect its speed of hydrolysis except slightly at the beginning of the hydrolysis, while toward the end of the hydrolysis the rate was decreased.

In all the hydrolyses, except that of the autoclaved solution of corn-starch, an insoluble white residue remained in the flask after conversion. This residue did not reduce Fehling's solution. Upon being hydrolyzed by concentrated hydrochloric acid, however, it reduced Fehling's solution. It was insoluble in water, alcohol, and ether, and gave a bluish violet coloration with iodine. It appeared as if the amount of this residue varied in different hydrolyses, depending upon the enzyme concentration and the substrate used. In order to determine whether or not this observation was correct, the weights of residues formed in the hydrolysis of potato and corn-starch were compared. 98 cc. of a 2 per cent solution of each starch (activants added) were hydrolyzed with 5 mg. of pancreatin. At the end of 24 hours, the amounts of sugar in the supernatant liquids were determined by analysis, after which the solutions were filtered through weighed Gooch crucibles, the residue washed several times with water, dried at 100°, and weighed. The weight of maltose formed from potato starch was 1.4240 gm., the weight of residue being 0.0113 gm. The corn-starch gave by the enzyme hydrolysis 1.2800 gm. of maltose, and an insoluble residue weighing 0.1602 gm. These residues were then hydrolyzed on a water bath for 5 hours with hydrochloric acid (sp. gr. 1.12). The large excess of acid was then neutralized by ammonium hydroxide, the solution filtered through asbestos, and the residue washed. The filtrate was collected in a 250 cc. volumetric flask and aliquots used for analysis. The dextrose formed was calculated to anhydrous maltose. The quantity of this sugar so obtained from the potato residue was 0.0156 gm., from the corn residue 0.1434 gm. (the residues were

obtained from 2 gm. of starch in each case). The total amount of maltose obtained from the potato starch (sum of maltose from acid and enzyme action) was 1.4396 gm., and from starch 1.4243 gm., the difference being only 0.0153 gm. If corn-starch was hydrolyzed by 100 mg. of enzyme, 1.7020 gm. of maltose were formed, while the weight of the residue was 0.0722 gm., showing that the weight of residue was dependent upon the enzyme concentration, or, in other words, that the residue was difficultly hydrolyzable. The data obtained are given in Table VII.

TABLE VII.

*Comparison of Maltose Formed after Acid Hydrolysis of Residues.*

Potato starch.		Corn-starch.	
	Maltose.		Maltose.
100 mg. of enzyme . . . .	1.7040 gm.	100 mg. of enzyme . . . . .	1.7020 gm.
85.02 per cent of total starch.		85.01 per cent of total starch.	

Mixed hydrolysis.

Maltose.		Maltose.	
	gm.		gm.
5 mg. of enzyme . . . .	1.4240	5 mg. of enzyme . . . . .	1.2800
Hydrolysis of residue. 0.0156		Hydrolysis of residue. . .	0.1434
Total . . . . .	1.4396	Total . . . . .	1.4234
71.98 per cent of total starch.		71.17 per cent of total starch.	

These data not only tend to demonstrate the fact that the maximum yield of maltose is approximately 85 per cent, but that the corn-starch appears to contain more of some substance difficultly hydrolyzed by enzymes, while the total amount of maltose formed from the enzyme hydrolysis of the starch and the acid hydrolysis of the residue is the same for each case.

Attention was now turned toward the salts which were used to accelerate the hydrolysis, namely sodium chloride and disodium phosphate, with the hope that possibly other activants would increase the speed of conversion to a greater extent than the salts named above. The effect of substituting 7 cc. of a fiftieth molar solution of various salts for the 7 cc. of fiftieth molar disodium phosphate, was determined, the salts used being sodium acetate,

sodium potassium tartrate, sodium citrate, sodium succinate, sodium ammonium hydrogen phosphate, monosodium phosphate,

TABLE VIII.

*Effect of Substituting Various Salts for Disodium Phosphate in Enzyme Hydrolysis of Corn-Starch.*

The mg. of maltose in 5 cc. of substrate are given at various intervals of time.

Time.	Na acetate.	NaK tartrate.	Na citrate.	Na succinate.	NaNH <sub>4</sub> -HPO <sub>4</sub>	Na <sub>2</sub> HPO <sub>4</sub>	Na <sub>2</sub> HPO <sub>4</sub> without NaCl.
hrs.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.50	3.7	4.2	5.0	5.0	5.4	5.7	0
2.00	14.2	10.8	24.5			22.6	
2.25						25.9	
2.50			29.3	24.7	28.7		1.7
4.00			38.2			37.1	
5.00						41.0	
5.50	33.8		47.6				
6.00				46.0	46.6		4.6
7.50		38.1	51.5			47.6	
21.00	59.9	56.0	59.6	59.9	57.3	58.9	19.2

TABLE IX.

*Effect of Substituting Various Salts for Sodium Chloride in Enzyme Hydrolysis of Corn-Starch.*

The maltose content per 5 cc. of substrate solution is given at various intervals of time.

Time.	No activants added.	KCl	NH <sub>4</sub> Cl	LiCl	NaCl (alone).
hrs.	mg.	mg.	mg.	mg.	mg.
0.50	2.50	6.4	5.4	5.4	5.7
2.50	5.00	30.9		20.8	19.8
3.50			36.5		
5.50		47.1	46.0		
6.00	10.00			36.5	41.5
21.00	32.1	58.3	56.7	52.7	58.3

0.00513 mol of sodium sulfate practically stopped the hydrolysis. At the end of 6.5 hours, 5 cc. of substrate contained but 4.2 mg. of maltose.

and disodium phosphate. In place of 300 mg. of sodium chloride equimolecular quantities (0.00513 mol) of the following salts were used together with the requisite amount of disodium phosphate:

potassium chloride, ammonium chloride, lithium chloride, and sodium sulfate. The sodium chloride and the phosphate were each tried alone. In all of the hydrolyses in which these salts were used, they were added after boiling the 2 gm. of corn-starch with 82 cc. of water under a reflux condenser. 1 mg. of pancreatin

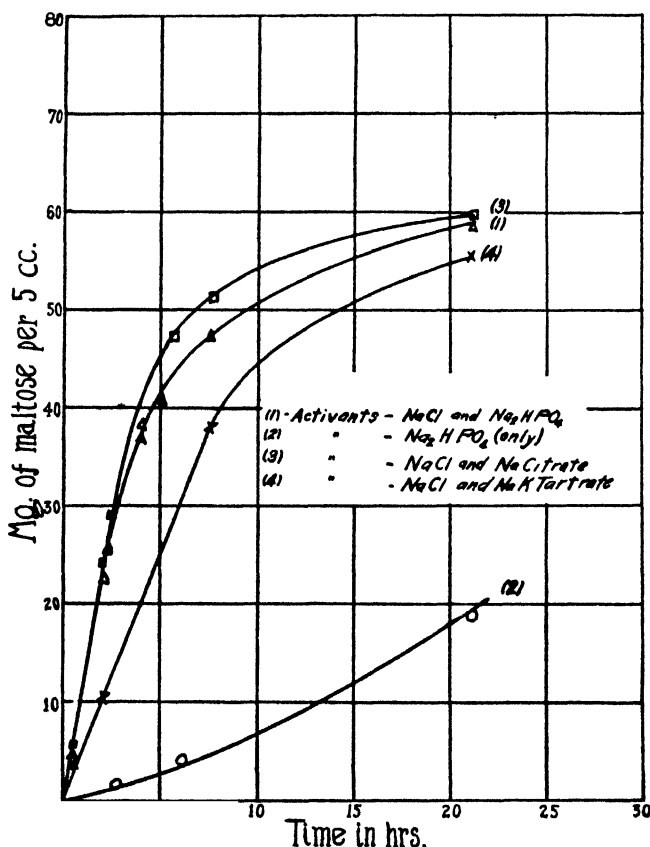


FIG. 3. Effect of different salts upon the rate of hydrolysis of corn-starch by 1 mg. of pancreatin.

was then added in 4 cc. from water containing the same concentration of salts at  $40^\circ$ , making a total volume of 98 cc. Table VIII gives the data obtained in studying the speed and extent of the hydrolyses where the substitutes mentioned above for the disodium phosphate were used

By comparing the results with those of the common activants, sodium chloride and disodium phosphate (next to the last column), the relative efficiencies of the other activants can be seen. Sodium acetate, sodium potassium tartrate, and disodium phosphate (this latter when used alone) are not as favorable to the enzymatic hydrolysis as the disodium phosphate; on the other hand, sodium succinate, microcosmic salt, and sodium citrate were as efficient as the disodium phosphate, sodium citrate being even a trifle better. The curves showing the influence of Rochelle salts, sodium citrate, and the phosphate when used alone as compared to the normal

TABLE X.

*Summary of Effect of Various Salts upon the Rate of Hydrolysis of Corn-Starch.*

Activants used.	Effect on hydrolysis.
KCl and Na <sub>2</sub> HPO <sub>4</sub> .	Same as NaCl and Na <sub>2</sub> HPO <sub>4</sub> .
NH <sub>4</sub> Cl " "	" " " " "
LiCl " "	Less than " " "
NaCl " NaK tartrate.	" " " " "
" " Na acetate.	Slightly less than NaCl and Na <sub>2</sub> HPO <sub>4</sub> .
" (only).	Less than NaCl and Na <sub>2</sub> HPO <sub>4</sub> .
" and NaH <sub>2</sub> PO <sub>4</sub> .	Much less than NaCl and Na <sub>2</sub> HPO <sub>4</sub> .
" " Na citrate.	Slightly greater than NaCl and Na <sub>2</sub> HPO <sub>4</sub> .
" " " succinate.	Same as NaCl and Na <sub>2</sub> HPO <sub>4</sub> .
" " microcosmic salt.	" " " " "
Na <sub>2</sub> HPO <sub>4</sub> (only).	Very little hydrolysis.
Na <sub>2</sub> SO <sub>4</sub> and Na <sub>2</sub> HPO <sub>4</sub> .	No hydrolysis.

curve are given in Fig. 3. The influence of the salts which were substituted for the sodium chloride are given in Table IX.

By comparing the results with those in which the sodium chloride was added with the disodium phosphate (sixth column, Table VIII) it will be seen that both potassium and ammonium chlorides were as favorable to enzymatic activity as the sodium chloride, while the lithium chloride and the sodium chloride (the latter when used without disodium phosphate) did not exert the same influence.

Fig. 4 shows the curves for the rate of conversion in which potassium and lithium chlorides were used, as well as the one in

which only the sodium chloride was added. Also the curve in which no activants were employed.

Table X shows the effect of all the salts used.

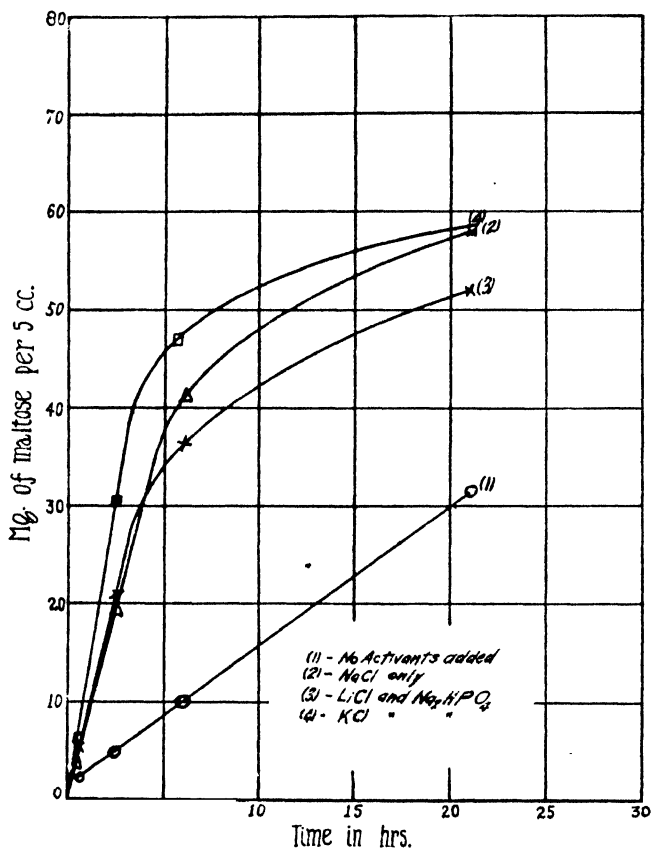


FIG. 4. Effect of different salts upon the rate of hydrolysis of corn-starch with 1 mg. of pancreatin.

#### SUMMARY.

1. Potato starch is hydrolyzed more rapidly than corn-starch by pancreatin, this being due to a difference in the chemical nature of the two, for the corn-starch was found to contain a material of a hemicellulose nature which was very slowly hydrolyzed by pancreatin.



2. The enzymatic action was found to stop when the maltose content was 85 per cent of the total weight of the starch, a fact which is in accord with the results of Sherman and his co-workers.

3. The rate of conversion of corn-starch was not increased after the starch had been rendered soluble according to Lintner, autoclaved dry, autoclaved in solution, frozen, or ether-extracted.

4. In an attempt to increase the speed of conversion of corn-starch by substituting other salts besides the disodium phosphate and sodium chloride, it was found that potassium and ammonium chlorides would act as well as the sodium chloride, while sodium citrate, sodium succinate, and sodium ammonium hydrogen phosphate could be used in place of the disodium phosphate. None of these salts increased the rate of hydrolysis more than the sodium chloride and phosphate except sodium citrate which, when used with the sodium chloride, showed a slightly more favorable effect.

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# THE COLORIMETRIC DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF MILK, WHEY, AND CREAM.

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## INTRODUCTION.

Since Foa (1) first reported the determination of the hydrogen ion concentration of cow's milk by the electrometric method, many others have carried out more extensive investigations. Most prominent among these investigations is the work of Van Slyke and Baker (2). They investigated 300 samples of fresh cow's milk and found them to fall between the pH limits of 6.5 to 7.2; 80 per cent of the samples fell between the limits 6.5 and 6.76.

The determination of the hydrogen ion concentration of milk by the colorimetric method offers considerable difficulty supposedly due to the marked turbidity of the milk itself. Five methods have been proposed to overcome this difficulty.

1. Baker and Van Slyke (3, 4) and Baker and Breed (5) added the indicator, brom-cresol purple, directly to the milk contained in test-tubes and compared the unknown samples with the color obtained with standard samples of normal milk, or with normal milk to which small amounts of acid or alkali had been added. The main difficulties in this method are the lack of definite color standards and the opaqueness of the milk, as the authors have pointed out.

2. Schultz and Chandler (6) dialyzed 1 cc. of milk against 2 cc. of neutral distilled water for 7½ minutes and determined the hydrogen ion concentration of the diffusate colorimetrically. They say, "Distilled water gave us the same results as physiological salt solution, and the proportion of milk to water used the same as equal volumes." Schultz, Marx, and Beaver (7) dialyzed 1 cc. of milk against 2 cc. of 0.8 per cent neutral salt solution for 5 minutes. Kramer and Greene (8) dialyzed 5 cc. of milk against an equal volume of 0.9 per cent NaCl solution for 5 minutes and then determined the hydrogen ion concentration of the diffusate. These investigators concluded that the colorimetric hydrogen ion concentration of the diffusate agreed very well with the hydrogen ion concentration of the original undialyzed milk determined by the electrometric method. This dialysis method had previously been applied to blood.

Kühne (9) in 1865 dialyzed blood and used the diffusate for determining its reaction. Later Levy, Rowntree, and Marriott (10), Scott (11), and Dale and Evans (12) used the dialysis through collodion sacs for determining the hydrogen ion concentration of blood.

3. Brown (13) recommends the dilution of the milk with water to decrease the effect of the turbidity; the amount of dilution was not stated exactly but it would probably be a dilution of about 1 part of milk to 15 or 20 parts of water. He states that dilution of milk with water does not change the pH. Murray and Weston (14) used cream diluted 1 to 5 for colorimetric determinations, but they point out the possibility of error due to such dilution and consider their results as comparative only among themselves. Murray and Weston used Medalia's (15) method of no buffer standards in which the relationship between color ratio and pH is based on a misconception. The drop ratio should be considered to be proportional to the hydrogen ion concentration as described by Gillespie (16-18). This may have caused a maximum error of about 0.25 pH in some of the standards used by Murray and Weston. Lisk (19) diluted milk 1 to 10 and argued that this dilution should have no effect on the hydrogen ion concentration. She does not state whether the electrometric or colorimetric method was used.

4. Sammis and Santschi (20) say that colorimetric determinations of the hydrogen ion concentration of milk usually cannot be made directly, because the casein adsorbs the color. They suggest that the casein be precipitated by rennet and that color comparisons be made on the whey. They say, "The fresh whey is said to have the same hydrogen ion content as the milk," but fail to state who made this statement. Experiments which we have made indicate that this statement may or may not be true, depending on the hydrogen ion concentration of the milk. Also it is known that whey shows indicator errors, so that the method suggested by Sammis and Santschi is of no value in determining true hydrogen ion concentration.

5. Taylor (21) added 2 drops of milk to 10 cc. of distilled water containing indicator in a test-tube and a corresponding number of drops of phosphate or citrate buffers to the same amount of water and indicator contained in other test-tubes. This method involves the assumption that the buffers change in hydrogen ion concentration on dilution to the same extent as does milk. This was not found to be the case in our experiments. In addition the dilution is carried so far that the quality of the distilled water and its hydrogen ion concentration would become a very important factor.

Brown (13) also devised a method for the determination of the hydrogen ion concentration of small amounts of fluid. The method consists essentially in using small cylinders about 3 mm. deep and holding about 10 drops for the containers for the comparison standards and the unknown. These cylinders are placed on an opal glass plate. The success in applying this method to turbid liquids depends on diluting the turbid liquid to minimize the effect of the turbidity. This dilution is justified only when it does not appreciably change the hydrogen ion concentration or when

the change in hydrogen ion concentration with dilution is known and corrected for. Brown found that dilution of several culture media to as little as one loop of the culture medium to 10 drops of water did not appreciably change the hydrogen ion concentration of the medium as determined colorimetrically. He says this dilution method is applicable to milk, although he gives no data showing the success of its use.

The dialysis method removes the difficulty of the turbidity and protein error of the milk but has as a possible source of error a Donnan equilibrium, a salt, and a dilution effect. The dilution method reduces to a considerable degree the turbidity but introduces the possibility of error due to the extensive dilution of the milk. That dilution of milk does alter the hydrogen ion concentration has been shown very clearly by Taylor (21) who found that on diluting fresh milk the solution became distinctly more alkaline, while on diluting sour milk the hydrogen ion concentration increased slightly. Our experiments indicate that this slight increase in hydrogen ion concentration found by Taylor after diluting sour milk was an error. Clark (22) presents data showing that diluting milk with distilled water lowers the hydrogen ion concentration appreciably, and Baker and Van Slyke (3) state that adding water to fresh milk causes a decrease in the hydrogen ion concentration. Tillmans and Obermeier (23) state that dilution with water has little effect on the hydrogen ion concentration. Numerous investigators have shown that dilution of milk causes a decrease in the titratable acidity.

#### EXPERIMENTAL.

##### *Study of the Dialysis Method.*

If the work of the investigators who show that milk becomes less acid on dilution is correct, we would expect the diffusate from dialyzed milk to be more alkaline than the original milk. Also if a Donnan equilibrium is set up across the collodion membrane, we would expect the diffusate to be more alkaline than the original milk. A series of dialysis experiments was carried out to test this point. Collodion bags were made in test-tubes and the dialysis was carried out in larger test-tubes; the levels of the milk in the bag and the water outside of the bag were made equal. The collodion bags held 25 cc. of milk. The milk was dialyzed against 25 cc. of distilled water for 30 minutes. Schultz and Chandler (6)

dialyzed 1 cc. of milk against 2 cc. of distilled water for  $7\frac{1}{2}$  minutes and Kramer and Greene (8) dialyzed 5 cc. of milk against 5 cc. of 0.9 per cent sodium chloride for 5 minutes. In our experiments we used larger amounts in order to have more material for the tests; we also dialyzed for a longer period of time in order to give the larger amounts of liquid more time to reach constant values. The experiments reported were carried out with milk which was fresh or nearly fresh, so that the development of lactic acid due to the growth of bacteria would not influence the results. The hydrogen ion concentration of the original milk, of the dialyzate, and of the diffusate was determined electrometrically.

In making the electrometric determinations, Bailey (24) electrodes were used. The electromotive force was measured on a type K potentiometer, using a type R galvanometer as a current detector. The equipment was kept in an air thermostat held at 25°C. The electromotive force readings were converted to pH values by means of the Schmidt and Hoagland (25) conversion tables.

Twenty samples of milk were examined; the pH of the dialyzate averaged 0.04 units higher than the pH of the original milk; while the pH of the diffusate averaged 0.13 pH higher than the pH of the original milk.

It was found later that the average value obtained for the hydrogen ion concentration of fresh milk diluted with an equal volume of distilled water was 0.13 pH more alkaline than the pH of the undiluted milk. This value is in exact agreement with the average increase in pH of the diffusate over that of the original milk. This accounts quantitatively on the basis of dilution for the higher pH of the diffusate but does not explain why the dialyzate did not also decrease in hydrogen ion concentration to the extent of 0.13 pH units instead of 0.04 pH.

The hydrogen ion concentration of the diffusate from some of these samples was determined colorimetrically by both the Brown (13) method and by the conventional test-tube comparator method using standardized buffer solutions. The buffer solutions were prepared according to Clark and Lubs (26) and were standardized electrometrically at 25°C. The results obtained are given in Table I. The colorimetric methods were found to agree with each other fairly well and gave results which were about 0.10 pH

more acid than they should have been according to the electrometric determinations on the diffusate. When we compare the determinations made on the diffusate with the electrometric determinations made on the original milk, we find that the diffusate averaged 0.13 pH more alkaline than the original milk by the electrometric method, and 0.03 pH and 0.04 pH more alkaline by the colorimetric methods. Thus our results are in agreement with the other investigators who found that the colorimetric determinations on the hydrogen ion concentration of the diffusate

TABLE I.

*Determination of Hydrogen Ion Concentration of Milk by Determining Hydrogen Ion Concentration of the Diffusate.*

Original milk.	Diffusate.			Difference from electrometric.		Difference of diffusate from original milk.		
	Electrometric.	Brown method.	Test-tube comparator.	Brown method.	Test-tube comparator.	Electrometric.	Brown method.	Test-tube comparator.
pH	pH	pH	pH	pH	pH	pH	pH	pH
6.46	6.53	6.4	6.4	-0.13	-0.13	+0.07	-0.06	-0.06
6.50	6.61	6.5	6.4	-0.11	-0.21	+0.11	0.00	-0.10
6.48	6.61	6.5	6.5	-0.11	-0.11	+0.13	+0.02	+0.02
6.63	6.70	6.65	6.6	-0.05	-0.10	+0.07	+0.02	-0.03
6.59	6.75	6.7	6.75	-0.05	0.00	+0.16	+0.11	+0.16
6.57	6.77	6.7	6.75	-0.07	-0.02	+0.20	+0.13	+0.18
6.62	6.79	6.6	6.7	-0.19	-0.09	+0.17	-0.02	+0.08
6.58	6.72	6.6	6.65	-0.12	-0.07	+0.14	+0.02	+0.07
Average.....				-0.10	-0.09	+0.13	+0.03	+0.04

from milk agreed with the electrometric determinations made on the original milk, yet our results indicate that this agreement is a fortunate coincidence in that the dilution error is just about compensated for by an indicator error. We used brom-thymol blue and brom-cresol purple for this range. Table I indicates that the dialysis method is fairly satisfactory for the colorimetric determination of the hydrogen ion concentration of milk if we assume that the two errors approximately compensate each other as they seem to do in our experiments, and we can, therefore, take the colorimetric determination of the hydrogen ion concentration of the diffusate as being about 0.03 to 0.04 pH greater than the pH

of the original milk. Our experiments have shown that if the hydrogen ion concentration of the milk is changing rapidly, due to the development of acid, then 30 minutes dialysis is a long enough period of time for the milk to change in hydrogen ion concentration during the dialysis. As mentioned before, Schultz and Chandler (6) recommend  $7\frac{1}{2}$  minutes dialysis and Kramer and Greene (8) recommend 5 minutes and both use smaller amounts of milk.

*Study of the Dilution Method.*

While the dialysis method offers no serious difficulty when used as a routine test, yet it necessitates a supply of dialyzers and, in addition, time is consumed by the dialysis. It would be a distinct advantage if the dialysis could be eliminated and thus do away with the necessity of preparing the dialyzers and also shorten the technique and time necessary to make a determination.

Brown (13) based his method on the condition that turbid liquids did not change in hydrogen ion concentration on dilution. He said that milk was such a liquid but presented no data to prove his statement. Brown did show that various culture media did not change appreciably in hydrogen ion concentration on dilution with water. We tried the effect of dilution on the hydrogen ion concentration, determined electrometrically, of two culture media making various dilutions up to 1 part of media to 19 parts of water. As the pH of the other dilutions fell in between the values for the undiluted media and the media diluted 1 to 19, only the values obtained for the highest dilution are given. The results are presented in Table II. It will be observed that the two samples of media which we investigated and which were near the neutral point did not change in hydrogen ion concentration on dilution. After adding acid to these media we found that they became more alkaline on dilution, and after adding alkali they became more acid on dilution. The effect of dilution was not great, the maximum change being 0.24 pH in these few experiments.

Taylor (21) found that fresh milk changed appreciably in hydrogen ion concentration on dilution with several volumes of water. He plotted the logarithms of the dilution against the pH and found that the points fell on a straight line. His results indicate that the hydrogen ion concentration of milk changes in a

uniform manner as it is diluted with water. This led us to study the constancy of this dilution factor and its variation with different samples of milk and with the hydrogen ion concentration of the milk diluted.

We first diluted several samples of milk with various amounts of distilled water and determined the hydrogen ion concentration of the dilutions. The points fell on the logarithmic straight line up through dilutions of 1 to 29, but a bending was indicated with dilutions of 1 to 49. Because of the possibility that a dilution of 30 volumes might not always give results which would fall on a straight line and because if the dilution was carried too far solutions

TABLE II.

*Effect on Hydrogen Ion Concentration, Determined Electrometrically, of Diluting Bacteriological Culture Media with Water.*

Culture medium.	Original hydrogen ion concentration.	Hydrogen ion concentration after diluting 1 to 19.	Difference.
	pH	pH	pH
Peptone water.....	7.09	7.09	0.00
“ “ + HCl.....	4.04	4.28	+0.24
“ “ + NaOH.....	8.68	8.50	-0.18
Nutrient bouillon.....	7.37	7.37	0.00
“ “ + HCl.....	6.67	6.76	+0.09
“ “ + NaOH.....	8.12	8.00	-0.12
“ “ + “.....	9.06	8.90	-0.16

might be obtained which were too weakly buffered to be reliable, we restricted ourselves in the main part of this investigation to dilutions up to 1 part of milk with 19 parts of water. Such dilutions are called in this paper dilutions to 20 volumes. Cullen (27) in his study of the use of diluted blood plasma for the determination of the hydrogen ion concentration of the blood colorimetrically chose a dilution with 20 volumes as the best. A bending of the curve was reached for dilutions around 20 volumes. Cullen, however, diluted with physiological salt solution. Hastings and Sendroy (28) also studied the dilution method. Hastings, Sendroy, and Robson (29) diluted urine 1 to 5 with distilled water for the colorimetric determination of hydrogen ion concentration. They found that the pH was increased by the dilution an average



of 0.08 pH units determined electrometrically. Their results on one sample show a progressive increase in pH on dilution. Muntwyler, Norris, and Myers (30) concluded the correction factor for a 1 to 5 dilution of urine was about 0.1 pH.

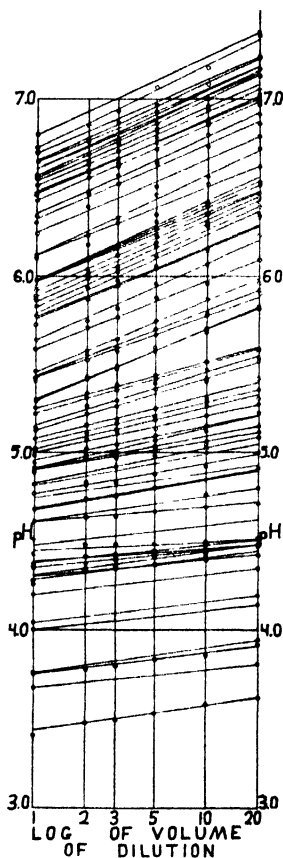


FIG. 1. Effect on the pH of diluting various samples of milk with different amounts of distilled water. 1 part of milk was made up to the volumes indicated, thus the 20 volume dilution means 1 volume of milk added to 19 volumes of water.

In studying the dilution factors the pH of the original milk and of the milk diluted to a total of 2, 3, 5, 10, and 20 volumes was plotted against the logarithm of the volume to which the milk was diluted, the best line was drawn through the points, and the differ-

ence in pH of the original milk and of the 20 volume dilution as indicated by the line was taken as the dilution factor. Some of the data as plotted are given in Fig. 1. In order to show more clearly the agreement between the actual hydrogen ion concentration of the milk and the hydrogen ion concentration of the milk as

TABLE III.

*Fresh Milk. Hydrogen Ion Concentration of Original Milk, of Original Milk as Indicated by Dilution Curve, of Milk Diluted to 20 Volumes, and the Difference Between Them.*

Laboratory No.	Raw or pasteurized.	Hydrogen ion concentration.			
		Whole milk, experimental.	Determined from curve.		
			Whole milk.	Diluted to 20 volumes.	Difference.
		pH	pH	pH	pH
69	Raw.	6.73	6.73	7.33	0.60
70	"	6.67	6.63	7.18	0.55
40	"	6.65	6.65	7.22	0.57
93	"	6.63	6.63	7.13	0.50
72	"	6.63	6.63	7.18	0.55
92	"	6.62	6.62	7.16	0.54
69	"	6.60	6.60	7.15	0.55
68	"	6.60	6.60	7.16	0.56
67	"	6.60	6.60	7.15	0.55
66	"	6.60	6.61	7.15	0.54
66	"	6.58	6.62	7.17	0.55
16	Pasteurized.	6.57	6.57	7.16	0.59
91	Raw.	6.57	6.60	7.06	0.46
71	"	6.56	6.55	7.10	0.55
57	"	6.55	6.54	7.08	0.54
70	"	6.55	6.55	7.05	0.50
85	"	6.55	6.63	7.13	0.50
67	"	6.54	6.55	7.12	0.57
26	Pasteurized.	6.54	6.54	7.08	0.54
66	Raw.	6.51	6.55	7.06	0.51

indicated by this line some of the data obtained are given in Tables III, IV, and V. These tables also give the hydrogen ion concentration indicated by this line for the dilution of the milk to 20 volumes together with the difference. This difference is the correction factor for dilution. Table III gives some of the data for fresh milk, Table IV for artificially soured milk, that is milk soured

by inoculation with commercial starters, and Table V gives some of the data for naturally soured milk. This study included mixed whole and skim milk from the Cornell Dairy herd, milk from individual cows in the herd, and patrons' milk brought in by dairymen in the vicinity. These tables show that the hydrogen ion con-

TABLE IV.

*\*Artificially Soured Milk. Hydrogen Ion Concentration of Original Milk, of Original Milk as Indicated by Dilution Curve, of Milk Diluted to 20 Volumes, and the Difference Between Them.*

Laboratory No.	Raw or pasteurized.	Hydrogen ion concentration.			
		Whole milk, experimental.	Determined from curve.		
			Whole milk.	Diluted to 20 volumes.	Difference.
		pH	pH	pH	pH
30	Pasteurized.	5.94	5.96	6.56	0.60
31	"	5.72	5.76	6.28	0.52
53	"	5.55	5.58	6.09	0.51
46	"	5.42	5.42	5.87	0.45
53	"	5.41	5.43	5.91	0.48
47	"	5.29	5.29	5.81	0.52
46	Raw.	5.25	5.25	5.59	0.34
104	"	5.07	5.07	5.39	0.32
47	Pasturized.	5.00	5.05	5.35	0.30
54	"	4.91	4.90	5.14	0.24
46	"	4.89	4.89	5.11	0.22
31	"	4.68	4.68	4.90	0.22
55	"	4.51	4.61	4.71	0.10
53	"	4.35	4.34	4.46	0.12
103	Raw.	4.35	4.35	4.46	0.11
104	"	4.28	4.30	4.47	0.17
43	"	4.26	4.30	4.42	0.12
103	"	3.75	3.75	3.91	0.16
103	"	3.41	3.44	3.62	0.18

centration of the original milk as indicated by the curves agrees very well with the hydrogen ion concentration found experimentally for the milk before dilution. Fig. 1 and Table III show that the slope of the dilution line for fresh milk of different hydrogen ion concentration is about the same. Dilution to 20 volumes caused a decrease in the hydrogen ion concentration of fresh milk of about 0.54 pH units.

TABLE V.

*Naturally Soured Milk. Hydrogen Ion Concentration of Original Milk, of Original Milk as Indicated by Dilution Curve, of Milk Diluted to 20 Volumes, and the Difference between Them.*

Laboratory No.	Raw or pasteurized.	Hydrogen ion concentration.			
		Whole milk, experimental.	Determined from curve.		
			Whole milk.	Diluted to 20 volumes.	Difference.
		<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
84	Raw.	6.47	6.47	7.01	0.54
58	Pasteurized.	6.45	6.45	7.00	0.55
85	Raw.	6.43	6.42	6.98	0.56
8	Pasteurized.	6.34	6.34	6.91	0.57
56	"	6.30	6.32	6.86	0.54
59	Raw.	6.24	6.24	6.78	0.54
42	"	6.12	6.12	6.62	0.50
40	"	6.10	6.12	6.71	0.59
38	Pasteurized.	6.01	6.01	6.45	0.44
35	"	6.01	6.01	6.45	0.44
42	Raw.	5.98	5.98	6.53	0.55
43	"	5.98	5.97	6.51	0.54
34	Pasteurized.	5.98	6.00	6.42	0.42
87	Raw.	5.96	5.96	6.51	0.55
60	"	5.96	5.92	6.47	0.55
56	Pasteurized.	5.95	5.98	6.54	0.56
87	Raw.	5.88	5.88	6.45	0.57
49	Pasteurized.	5.86	5.85	6.42	0.57
10	"	5.82	5.82	6.39	0.57
48	"	5.80	5.78	6.36	0.58
12	"	5.77	5.75	6.28	0.53
48	"	5.63	5.63	6.20	0.57
62	"	5.46	5.45	5.98	0.43
63	"	5.30	5.30	5.83	0.53
60	Raw.	5.21	5.22	5.58	0.36
14	Pasteurized.	5.14	5.15	5.54	0.39
48	"	5.12	5.12	5.50	0.38
62	"	5.02	5.02	5.32	0.30
60	"	4.94	4.95	5.20	0.25
48	"	4.90	4.91	5.20	0.29
60	"	4.82	4.83	5.08	0.25
49	"	4.77	4.76	4.98	0.22
62	"	4.67	4.77	4.85	0.08
50	"	4.43	4.43	4.48	0.05
36	"	4.37	4.40	4.50	0.10
103	Raw.	4.35	4.35	4.46	0.11

As the milk sours and after reaching a pH of about 5.6, then the decrease in acidity on dilution becomes less. Tables IV and V indicate that the rate of decrease in the dilution factor with increasing acidity is the same whether souring is produced by the organisms naturally present in the milk, or by the addition of commercial starters. In cases where the milk had become so sour that the casein had coagulated, it was necessary to shake the samples vigorously in order to break up the curd so that in making the dilutions all parts of the milk were diluted in proportion. If this precaution was not observed, the results were not uniform. It will be shown later that the dilution factor for whey is not the same as for the milk from which the whey was obtained.

The data obtained indicate that the dilution factor is a function of the hydrogen ion concentration of the original milk. The dilution factors for 158 samples of milk were obtained by plotting curves, a part of which are given in Fig. 1. These dilution factors were then plotted against the hydrogen ion concentration of the diluted milk (20 volume dilution). The results of this plotting are found in Fig. 2. The results were plotted in this way so that one could determine the hydrogen ion concentration of a sample of milk diluted to 20 volumes and then read from the curve the fraction of a pH unit to be subtracted from the reading obtained with the 20 volume dilution to give the pH of the original undiluted sample. It will be observed that in some instances the data for milk plotted in Fig. 2 do not fall very close to the line, but in about 82 per cent of the samples the error in the correction factor is not greater than 0.05 pH and in 98 per cent of the cases it is not greater than 0.10 pH. The fact that the points in Fig. 2 do not all fall exactly on the line was to be expected and is probably due to differences in the composition of the milk and especially differences in electrolyte and casein content. Apparently, fresh milk from cows giving extremely large amounts of milk seems to change slightly less on dilution to 20 volumes than does milk from cows giving smaller amounts.

A similar series of experiments on the effect on the hydrogen ion concentration of diluting whey was carried out. Whey was prepared from fresh milk by the addition of rennet. In the more acid regions wheys from naturally soured milk, from artificially soured milk, and from rennet were investigated. Just as with milk so

also with whey, the logarithms of the dilutions plotted against the pH gave straight lines. The angle of these lines with the abscissa was not as great in the case of the rennet whey from fresh milk as was the slope of the line found by diluting the milk itself. On the other hand the whey from soured milk had a slightly steeper dilution slope than was obtained by diluting the thoroughly shaken milk itself. The change in hydrogen ion concentration of diluting whey to 20 volumes was plotted against the pH of the whey diluted to 20 volumes in Fig. 2. It will be observed that the

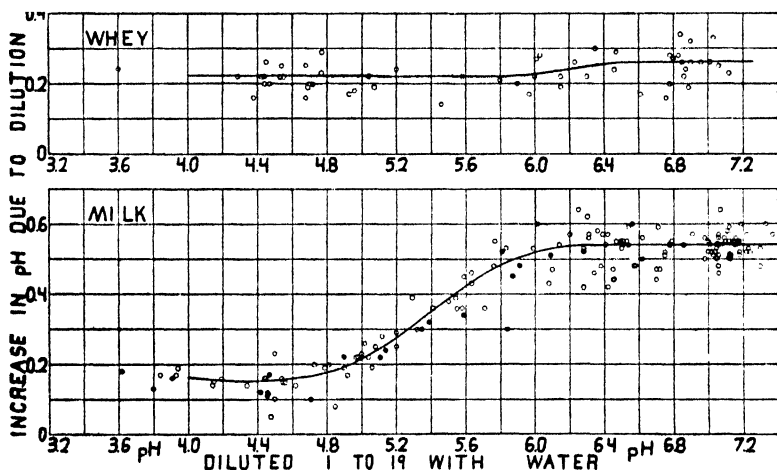


FIG. 2. Increase in pH caused by diluting milk or whey with 19 volumes of distilled water plotted against the pH of the diluted milk or whey. The circles indicate the fresh samples and samples which were soured naturally; the dots indicate the samples soured by the addition of commercial starters.

line is nearly straight, the original hydrogen ion concentration of the whey having little effect on the decrease in acidity due to dilution. The more acid wheys did, however, decrease in acidity slightly less than the whey from fresh milk. There are 55 points plotted in this curve for the dilution of whey; none of them is further away from the curve than 0.10 pH and 78 per cent of the points are within 0.05 pH of the curve or closer.

With the use of Fig. 2 it is possible to construct a table of correction factors for the effect of dilution to 20 volumes with distilled water on the hydrogen ion concentration of milk and whey. Table

TABLE VI.

*Table of Correction Factors for Colorimetric Determination of Hydrogen Ion Concentration of Milk and Whey by Dilution Method.*

The product is diluted 1 to 19 with distilled water and the colorimetric determination made in the conventional way with turbidity blank. The colorimetric pH reading is located in the first column, then the pH of the undiluted milk or whey is found in the corresponding row.

Colorimetric reading.	Whole milk and skim milk.		Whey.		Indicator.
	Correction factor.	Original undiluted.	Correction factor.	Original undiluted.	
<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	
7.4	0.54	6.86			Phenol red.
7.3	0.54	6.76			
7.2	0.54	6.66	0.26	6.94	
7.1	0.54	6.56	0.26	6.84	
7.0	0.54	6.46	0.26	6.74	
6.9	0.54	6.36	0.26	6.64	
6.8	0.54	6.26	0.26	6.54	
6.7	0.54	6.16	0.26	6.44	
6.6	0.54	6.06	0.26	6.34	Brom-cresol purple.
6.5	0.54	5.96	0.26	6.24	
6.4	0.54	5.86	0.25	6.15	
6.3	0.54	5.76	0.25	6.05	
6.2	0.54	5.66	0.24	5.96	Chlor-phenol red.
6.1	0.53	5.57	0.23	5.87	
6.0	0.52	5.48	0.23	5.77	
5.9	0.50	5.40	0.22	5.68	
5.8	0.48	5.32	0.22	5.58	
5.7	0.46	5.24	0.22	5.48	
5.6	0.43	5.17	0.22	5.38	
5.5	0.39	5.11	0.22	5.28	
5.4	0.35	5.05	0.22	5.18	
5.3	0.31	4.99	0.22	5.08	
5.2	0.28	4.92	0.22	4.98	
5.1	0.24	4.86	0.22	4.88	
5.0	0.22	4.78	0.22	4.78	
4.9	0.19	4.71	0.22	4.68	Brom-cresol green.
4.8	0.18	4.62	0.22	4.58	
4.7	0.17	4.53	0.22	4.48	
4.6	0.16	4.44	0.22	4.38	
4.5	0.16	4.34	0.22	4.28	
4.4	0.15	4.25	0.22	4.18	Brom-phenol blue.
4.3	0.15	4.15	0.22	4.08	
4.2(?)	0.15	4.05	0.22	3.98	
4.1(?)	0.16	3.94	0.22	3.88	
4.0(?)	0.16	3.84	0.22	3.78	

VI is such a table. By the use of this table it should be possible to determine the hydrogen ion concentration of milk or whey diluted to 20 volumes, and by subtracting the proper correction factor tell the hydrogen ion concentration of the original product with an error of 0.05 pH or less in about 80 per cent of the cases and an error of 0.10 pH or less in about 98 per cent of the cases.

All of the work on dilution reported thus far has been done electrometrically. It remains to be shown how accurately the colorimetric determination of the hydrogen ion concentration of the diluted milk can be made to indicate the hydrogen ion concentration of the original undiluted milk by the application of the dilution factors given in Table VI.

*Whole and Skim Milk.*—The colorimetric determinations were made with standard buffer solutions using a test-tube comparator and a turbidity blank. The test-tubes used had an internal diameter of 13 mm. In such test-tubes a 20 volume dilution of milk permitted considerable light to pass through when the tubes were held before a strong light. Most of the color comparisons were made with artificial light produced by an electric light held behind a sheet of thin white paper. Artificial light was used because on cloudy days the natural light was not intense enough to penetrate the turbidity of the solution. In some cases the tints with the artificial light were different from those obtained with direct sunlight: brom-phenol blue showed the greatest difference, the tints being red with artificial light and blue with sunlight. With certain of the indicators pronounced errors were observed: the error with brom-thymol blue amounted to 0.5 to 0.7 pH, methyl red also showed a considerable error. As a result of the study of a large number of samples the indicators best adapted for the various ranges of pH were selected and are given in Table VI.

A large number of samples of milk of various hydrogen ion concentrations was diluted to 20 volumes and the hydrogen ion concentration of the diluted milk was determined both electrometrically and colorimetrically. The hydrogen ion concentration of the original milk was determined electrometrically. Some of the results obtained after eliminating those which did not conform to the indicators and range indicated in Table VI are given in Table VII.



TABLE VII.

*Whole Milk. Showing Accuracy with Which Colorimetric and Electrometric Determination of Hydrogen Ion Concentration of Milk Diluted to 20 Volumes Indicates, after Correction, the Hydrogen Ion Concentration of Undiluted Milk.*

Original milk.	Milk diluted to 20 volumes.		Corrected for dilution should give pH of undiluted milk.			
	Electrometric.	Colorimetric.	Electrometric.	Colorimetric.	Error.	
					Electrometric.	Colorimetric.
pH	pH	pH	pH	pH	pH	pH
6.63	7.13	7.1	6.59	6.56	-0.04	-0.07
6.62	7.13	7.2	6.59	6.66	-0.03	+0.04
6.59	7.19	7.2	6.65	6.66	+0.06	+0.07
6.58	7.06	7.0	6.52	6.46	-0.06	-0.12
6.57	7.12	7.1	6.58	6.56	+0.01	-0.01
6.56	6.95	7.1	6.41	6.56	-0.15	0.00
6.53	7.08	6.95	6.54	6.41	+0.01	-0.12
6.53	7.11	7.1	6.57	6.56	+0.04	+0.03
6.52	7.03	6.9	6.49	6.36	-0.03	-0.16
6.52	7.04	7.1	6.50	6.56	-0.02	+0.04
6.50	7.06	7.1	6.52	6.56	+0.02	+0.06
6.49	7.06	7.1	6.52	6.56	+0.03	+0.07
6.48	6.98	7.0	6.44	6.46	-0.04	-0.02
6.48	7.05	7.0	6.51	6.46	+0.03	-0.02
6.48	7.00	7.0	6.46	6.46	-0.02	-0.02
6.43	7.07	6.9	6.53	6.36	+0.10	-0.07
6.26	6.70	6.75	6.16	6.21	-0.10	-0.05
6.25	6.72	6.9	6.18	6.36	-0.07	+0.11
6.24	6.79	6.7	6.25	6.16	+0.01	-0.08
6.23	6.75	6.8	6.21	6.26	-0.02	+0.03
6.23	6.70	6.8	6.16	6.26	-0.07	+0.03
6.21	6.75	6.7	6.21	6.16	0.00	-0.05
6.10	6.58	6.6	6.04	6.06	-0.06	-0.04
6.10	6.58	6.7	6.04	6.16	-0.06	+0.06
6.06	6.62	6.6	6.08	6.06	+0.02	0.00
5.97	6.50	6.5	5.96	5.96	-0.01	-0.01
5.95	6.54	6.5	6.00	5.96	+0.03	+0.01
5.95	6.50	6.5	5.96	5.96	+0.01	+0.01
5.90	6.36	6.4	5.82	5.86	-0.08	-0.04
5.90	6.38	6.35	5.84	5.81	-0.06	-0.09
5.87	6.41	6.5	5.87	5.96	0.00	+0.09
5.88	6.30	6.4	5.76	5.86	+0.08	+0.18
5.63	6.10	6.0	5.57	5.48	-0.06	-0.15
5.46	5.99	5.9	5.47	5.40	+0.01	-0.06

TABLE VII—*Concluded.*

Original milk.	Milk diluted to 20 volumes.		Corrected for dilution should give pH of undiluted milk.			
	Electro-metric.	Colori-metric.	Electro-metric.	Colori-metric.	Error.	
					Electro-metric.	Colori-metric.
<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
5.41	6.01	5.7	5.49	5.24	+0.08	-0.17
5.35	5.71	5.7	5.25	5.24	-0.10	-0.11
5.28	5.76	5.7	5.30	5.24	+0.02	-0.04
5.22	5.77	5.7	5.31	5.24	+0.09	+0.02
5.17	5.63	5.5	5.20	5.11	+0.03	-0.06
4.90	5.29	5.2	4.98	4.92	+0.08	+0.02
4.84	5.12	5.1	4.88	4.86	+0.04	+0.02
4.80	5.06	5.1	4.84	4.86	+0.04	+0.06
4.76	5.02	5.0	4.82	4.78	+0.06	+0.02
4.27	4.50	4.5	4.34	4.34	+0.07	+0.07
Average error taking into account the signs.....					-0.002	-0.012
" " disregarding the signs.....					0.047	0.059

In using Table VI it is important to use the indicators only in the range given in this table, for although the indicators may give color changes outside of the range given in the table for ordinary colorimetric determinations, yet we found that with diluted milk it frequently happened that the true pH value was not indicated. An example of this was found in the case of brom-cresol purple whose range is usually given as from pH 5.4 to 7.0, yet we found it best to restrict its range with the diluted milk samples to pH 6.3 to 6.6. Diluted milk samples with a pH value of 6.7 are rather hard to determine. Such samples read this pH with both phenol red and brom-cresol purple, but solutions whose true pH value is 7.0 frequently read 6.7 with brom-cresol purple, while phenol red gives the true value 7.0. For this reason, it is necessary to push the use of phenol red down nearer its lower pH limit than would otherwise be desirable.

Table VII lists 44 samples of milk. The hydrogen ion concentration of the dilutions was corrected back to the hydrogen ion concentration of the original milk by the use of the factors given in Table VI, and these values were compared with the actual hydrogen ion concentration of the undiluted milk. The errors in terms

of pH made in determining the hydrogen ion of the original milk from the 20 volume dilution are given in the last two columns. The maximum error made in the electrometric determinations was 0.15 pH, the average error disregarding signs was 0.047 pH, and the average error taking into account the signs was  $-0.002$  pH. The maximum error in the colorimetric determinations was 0.18 pH, the average error disregarding signs was 0.059 pH, and the average error taking into account the signs was  $-0.012$  pH. The average error taking into account the signs indicates that the average correction factors given in Table VI, as obtained from the line in Fig. 2, are approximately correct. The agreement between the average values for the electrometric and colorimetric determinations differs only by approximately 0.01 pH. This agreement seems particularly good in view of the fact that the buffer standards were prepared only for 0.2 pH intervals and most of the color determinations were read only to even tenths of pH.

*Whey.*—A study was made of the accuracy with which the hydrogen ion concentration of whey could be determined colorimetrically by the dilution method. Although the turbidity of the undiluted whey is not so great as to prevent the determination of the hydrogen ion concentration directly with the use of a turbidity blank, yet the undiluted whey gave indicator errors.

Clark, Zoller, Dahlberg, and Weimar (31) found that methyl red showed a considerable error when applied to whey; the reading with the indicator was pH 4.6 when the true reaction was pH 4.1. They also show that the indicator error is not a constant pH difference. We found that methyl red still gave an error when added to diluted whey. The two indicators brom-cresol green and brom-phenol blue which cover the same range of pH as methyl red were more satisfactory, although brom-phenol blue still gave an error with diluted whey, the reading being too low.

Results obtained with the dilution method applied to whey are given in Table VIII. The maximum error in determining electrometrically the hydrogen ion concentration of the whey from the hydrogen ion concentration of the whey diluted to 20 volumes was 0.10 pH, the average error taking into account the signs was  $-0.002$  pH, and the average error disregarding the signs was 0.033 pH. Using the colorimetric method the maximum error was 0.12 pH, the average error taking into account the signs was

-0.007 pH, and the average error disregarding the signs was 0.055 pH. A 20 volume dilution of whey reduces the turbidity so that it interferes hardly at all. There is one precaution to be

TABLE VIII.

*Whey. Showing Accuracy with Which Colorimetric and Electrometric Determination of Hydrogen Ion Concentration of Whey Diluted to 20 Volumes Indicates, after Correction, the Hydrogen Ion Concentration of Undiluted Whey.*

Original whey.	Whey diluted to 20 volumes.		Corrected for dilution should give pH of undiluted whey.			
	Electrometric.	Colorimetric.	Electrometric.	Colorimetric.	Error.	
					Electrometric.	Colorimetric.
pH	pH	pH	pH	pH	pH	pH
6.55	6.83	6.75	6.57	6.49	+0.02	-0.06
6.53	6.80	6.9	6.54	6.64	+0.01	+0.11
6.47	6.83	6.7	6.57	6.44	+0.10	-0.03
6.08	6.30	6.3	6.05	6.05	-0.05	-0.05
5.97	6.23	6.3	5.99	6.05	+0.02	+0.08
5.80	5.97	6.1	5.74	5.87	-0.06	+0.07
5.79	6.00	5.9	5.77	5.68	-0.02	-0.11
5.59	5.80	5.75	5.58	5.53	-0.01	-0.06
5.36	5.58	5.6	5.36	5.38	0.00	+0.02
5.32	5.46	5.5	5.24	5.28	-0.08	-0.04
4.82	5.04	5.0	4.82	4.78	0.00	-0.04
4.78	4.96	5.0	4.74	4.78	-0.04	0.00
4.76	4.93	5.1	4.71	4.88	-0.05	+0.12
4.50	4.70	4.7	4.48	4.48	-0.02	-0.02
4.47	4.77	4.7	4.55	4.48	+0.08	+0.01
4.43	4.68	4.55	4.46	4.33	+0.03	-0.10
4.27	4.47	4.4	4.25	4.18	-0.02	-0.09
4.22	4.44	4.5	4.22	4.28	0.00	+0.06
4.20	4.42	4.4	4.20	4.18	0.00	-0.02
4.17	4.43	4.4	4.21	4.18	+0.04	+0.01
Average error taking into account the signs.....					-0.002	-0.007
" " disregarding the signs.....					0.033	0.055

observed, however; that is, the correction factors are not accurate if the casein is only partially precipitated. In all of our experiments the hydrogen ion concentration of the whey was determined immediately after separating it from the curd.

The effect of dilution on the hydrogen ion concentration of a number of milk products was investigated.

*Heated Milk.*—While heating milk to the boiling point for 30 minutes increased the hydrogen ion concentration of milk even when the loss of water was prevented, yet such milk showed approximately the same dilution corrections as normal milk.

*Cream.*—Cream showed some peculiarities on dilution which were not exhibited by the other products. Baker and Van Slyke

TABLE IX.

*Effect of Dilution of Cream and Skim Milk Obtained from It, on Hydrogen Ion Concentration.*

Volume of dilution.	25 per cent cream.		40 per cent cream.	
	Cream.	Skim milk.	Cream.	Skim milk.
	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
	Sample 1.		Sample 3.	
1	6.51	6.50	6.39	6.67
2	6.70	6.63	6.83	6.80
3	6.77	6.71	6.92	6.88
5	6.90	6.77	7.04	6.96
10	6.99	6.91	7.12	7.09
20	7.10	7.04	7.27	7.19
	Sample 2.		Sample 4.	
1	6.58	6.58	6.56	6.66
2	6.77	6.72	6.83	6.81
3	6.86	6.77	6.90	6.84
5	6.95	6.88	6.98	6.96
10	7.09	6.97	7.12	7.05
20	7.19	7.12	7.22	7.19

(3) state that the hydrogen ion concentration increases with the fat content. We studied the effect of diluting 25 per cent and 40 per cent creams and the skim milk prepared from them. Typical examples of the data obtained are given in Table IX and are expressed graphically in Fig. 3. We found that the hydrogen ion concentration of the 40 per cent cream was distinctly higher than the skim milk, but on plotting the dilutions we found that they were less acid than the skimmed milk.

It would seem more logical that the cream and the skim milk

should have the same hydrogen ion concentration, than that they should differ, because in determining the hydrogen ion concentration of the cream one should actually be measuring the hydrogen ion concentration of the plasma, that is, the skim milk. If the fat merely occupies space in the plasma, it should make no difference in the hydrogen ion concentration whether the fat were present or not. As mentioned, the dilutions of cream were found to be less acid than the skim milk and the extrapolation of the line of the points indicates that the cream was slightly less acid than the skim milk. If we assume that in diluting the cream the only component

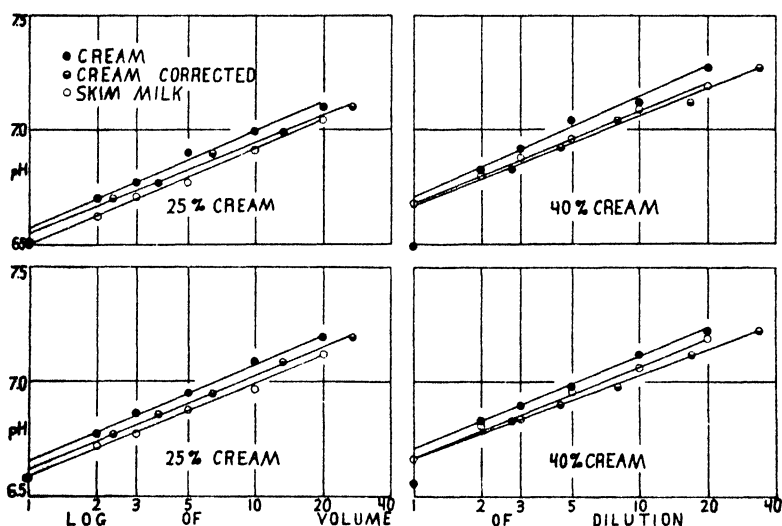


FIG. 3. Effect on the pH of diluting cream and the skim milk obtained from it with different amounts of distilled water.

which affects the pH by such dilution is the skim milk, then in diluting 40 per cent cream with an equal volume, etc., of water, the plasma present is actually being diluted at a faster rate than if the fat were absent. This would tend to account for the fact that the dilutions of the cream were more alkaline than the skim milk. Taking this into account, we find that the corrected dilution curves for the cream and the skim milk more nearly coincide, and in the case of the 40 per cent creams the extrapolation of the cream dilution curve to the whole cream line indicates that the cream has the same hydrogen ion concentration as the skim milk.

This indicates that the electrometric determination of the hydrogen ion concentration of high fat cream is actually in error and the true value can be obtained by extrapolating the dilution curve as mentioned above, or by making a determination on the skim milk. In determining the hydrogen ion concentration of high fat cream electrometrically by the time the mixture is saturated with hydrogen, the determination is not being made on cream but on buttermilk.

In Fig. 3 the cream dilution curve after correction does not exactly coincide with the skim milk dilution curve, indicating that perhaps the fat does exert some slight effect. The corrected curves for the 40 per cent cream lie under the curve for the skim milk while the corrected curves for the 25 per cent cream lie above the skim milk curve.

Our data indicate that a correct hydrogen ion determination of cream can be made electrometrically if the fat content of the cream is not too high. Thus the hydrogen ion concentrations of the 25 per cent creams determined electrometrically are in agreement with the hydrogen ion concentration of the skim milk. Also the hydrogen ion concentration of the 40 per cent cream diluted with an equal volume of water and corrected is in good agreement with the skim milk dilution curve.

The dilution method when applied to cream did not yield itself to accurate determinations colorimetrically. This was due to the turbidity caused by the large amounts of fat. In attempting to determine the hydrogen ion concentration of cream colorimetrically, results of about the same accuracy as those obtained with milk should be obtained by separating the skim milk and then diluting it to 20 volumes and applying the correction factors for milk as given in Table VI. The discussion of Table IX and Fig. 3 indicates that this procedure is justified. The skimmed milk can be separated from the cream by means of an ordinary laboratory centrifuge. If the cream has developed such an acidity that casein is thrown out in the centrifuge then the correction factors, as given in Table VI, do not apply.

*Milk Powder.*—In determining the hydrogen ion concentration of milk powder by the dilution colorimetric method, reconstituted milk was prepared from the product. The reconstituted milks were so prepared that they contained about the same amount of

solids not fat as is found in normal milk. The milk powders were obtained from several manufacturers. The correction factors given in Table VI were applied. The results obtained are given in Table X. The last four samples reported in Table X were reconstituted powdered milks which were allowed to develop a slight acidity before testing. This table indicates that the results obtained in applying the colorimetric method to reconstituted powdered milk are as satisfactory as the results obtained with normal whole milk.

TABLE X.

*Milk Powder. Hydrogen Ion Concentration of Reconstituted Milk Made from Milk Powder, and Application of Colorimetric Dilution Method of Determining Hydrogen Ion Concentration.*

Original product.	Diluted to 20 volumes.		Corrected for dilution by factors from Table VI.			
	Electro-metric.	Colori-metric.	Electro-metric.	Colori-metric.	Error.	
					Electro-metric.	Colori-metric.
<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
6.60	7.15	7.1	6.61	6.56	+0.01	-0.04
6.57	7.06	7.1	6.52	6.56	-0.05	-0.01
6.56	7.03	7.1	6.49	6.56	-0.07	0.00
6.56	6.96	7.1	6.42	6.56	-0.14	0.00
6.47	6.91	7.1	6.37	6.56	-0.10	+0.09
6.44*	6.94	6.9	6.40	6.36	-0.04	-0.08
6.30*	6.89	6.75	6.35	6.21	+0.05	-0.09
6.15*	6.72	6.70	6.18	6.16	+0.03	+0.01
5.63*	6.17	6.3	5.63	5.76	0.00	+0.13

\* Slight acidity allowed to develop.

*Evaporated Milk.*—Samples of evaporated milk were obtained directly from the manufacturers and also a few samples were purchased in the local market. In making the hydrogen ion determinations on these samples, the milk was approximately reconstituted before making the dilution to 20 volumes; that is, the evaporated milk was diluted with an equal volume of water to give a milk of approximately normal composition. The correction factors given in Table VI were applied. The results obtained for the hydrogen ion concentration are given in Table XI. This table indicates that the correction factors as given in Table VI are slightly too high.



*Sweetened Condensed Milk.*—The samples were obtained directly from the manufacturers and from local grocery stores. In this case also the milk was reconstituted by adding an equal volume of water before making the determinations. The correction factors, as given in Table VI, should not apply here. The reconstituted milk containing about the normal percentage of milk solids should contain about 20 to 25 per cent of sucrose; this should produce the same effect as was observed in cream; that is, in diluting 1 to 19 the milk plasma is being diluted at a faster rate than if the sugar were absent. If we make the assumption that the hydrated sugar

TABLE XI.

*Evaporated Milk. Hydrogen Ion Concentration of Evaporated Milk Diluted 1 to 1 Given in First Column, and Application of Colorimetric Dilution Method of Determining the Hydrogen Ion Concentration.*

Original product.	Diluted to 20 volumes.		Corrected for dilution by factors from Table VI.			
	Electro-metric.	Colori-metric.	Electro-metric.	Colori-metric.	Error.	
					Electro-metric.	Colori-metric.
pH	pH	pH	pH	pH	pH	pH
6.25	6.71	6.7	6.17	6.16	-0.08	-0.09
6.23	6.70	6.7	6.16	6.16	-0.07	-0.07
6.23	6.71	6.7	6.17	6.16	-0.06	-0.07
6.20	6.59	6.7	6.05	6.16	-0.15	-0.04
6.20	6.70	6.7	6.16	6.16	-0.04	-0.04
6.17	6.61	6.7	6.07	6.16	-0.10	-0.01
6.13*	6.63	6.7	6.09	6.16	-0.04	+0.03

\* Slight acidity allowed to develop.

occupies 25 per cent of the volume, the correction factor instead of being 0.54 pH units should be 0.60 pH units. Using this correction factor the electrometric results give the original pH with a mean error of 0.037 pH. The results of applying the hydrogen ion dilution method are given in Table XII. The sweetened condensed milk shows an indicator error with phenol red. From Table XII the correction factor for sweetened condensed milk based on the colorimetric determinations was found to be 0.85 pH instead of 0.60 pH for the electrometric determinations, or 0.54 pH as given in Table VI. The last two samples given in

TABLE XII.]

*Sweetened Condensed Milk. Hydrogen Ion Concentration of Sweetened Condensed Milk Diluted 1 to 1, and Application of Colorimetric Dilution Method of Determining of Hydrogen Ion Concentration.*

Original product.	Diluted to 20 volumes.		Corrected for dilution.			
	Electro-metric.	Colori-metric.	Factor 0.60 electro-metric.	Factor 0.85 colori-metric.	Error.	
					Electro-metric.	Colori-metric.
<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
6.48	7.16	7.35	6.56	6.50	+0.08	+0.02
6.48	7.08	7.3	6.48	6.45	0.00	-0.03
6.48	7.02	7.3	6.42	6.45	-0.06	-0.03
6.46	7.03	7.2	6.43	6.35	-0.03	-0.11
6.45	7.08	7.3	6.48	6.45	+0.03	0.00
6.41	7.00	7.3	6.40	6.45	-0.01	+0.04
6.30*	6.94	7.15	6.34	6.30	+0.04	0.00
6.09*	6.73	7.0	6.13	6.15	+0.04	+0.06
6.04*	6.58	6.9	5.98	6.05	-0.06	+0.01
6.44†	7.18	7.3	6.58	6.45	+0.14	+0.01
6.42†	7.16	7.35	6.56	6.50	+0.14	+0.08

\* Sample very dark.

† Sample kept in a warm place in the laboratory for several months.

TABLE XIII.

*Effect of Diluting Evaporated Milk and Sweetened Condensed Milk 1 to 1, on Hydrogen Ion Concentration. Determinations Made Electrometrically.*

Evaporated milk.			Sweetened condensed milk.		
Original.	1 to 1 dilution.	Difference.	Original.	1 to 1 dilution.	Difference.
<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
6.31	6.48	0.17	6.22	6.37	0.15
6.19	6.32	0.13	6.20	6.48	0.28
6.10	6.22	0.12	6.20	6.48	0.28
6.08	6.20	0.12	6.20	6.48	0.28
6.07	6.20	0.13	6.15	6.46	0.31
6.07	6.20	0.13	6.11	6.45	0.34
6.05	6.20	0.15	6.09	6.48	0.39
6.05	6.18	0.13	5.74	6.09	0.35
6.05	6.18	0.13			
5.86	5.98	0.12			
Average difference.....0.133			Average difference.....0.297		

Table XII were two samples which had been sitting in sealed cans for some months in a rather warm place in the laboratory.

As previously mentioned, the data in Tables XI and XII for evaporated and sweetened condensed milk were obtained by first diluting the sample 1 to 1 with distilled water. In order to show the effect of diluting these products 1 to 1 with distilled water the hydrogen ion concentration was determined on a series of samples both before and after making such a dilution. The results are given in Table XIII. It was to be expected that the dilution effect would be greater with the sweetened condensed milk than with the evaporated milk. Sweetened condensed milk is rather viscous at room temperature and it is difficult to saturate the electrodes and solution with hydrogen and there is a tendency for the determinations on the undiluted material to read a little low.

#### DISCUSSION.

The colorimetric method for determining hydrogen ion concentration is usually found to be most useful when results of extreme accuracy are not required. The standards are most frequently prepared at 0.2 pH intervals. Such standards permit readings to be made which are usually accurate to the nearest 0.1 pH. This is about the accuracy which can ordinarily be expected with the dilution method described in this paper. It seemed to us that the dilution method with the factors for dilution determined should offer the most satisfactory colorimetric method for milk of the five methods which have been proposed in the literature. It is possible that after the corrections for the dialysis method have been worked out it would yield more accurate results than the dilution method, but since the dialysis method necessitates a supply of dialyzers and requires more time, it was decided that the dilution method would probably prove to be more generally useful.

In the light of our work the following procedure for carrying out the dilution method would seem to be the most satisfactory. We have tried to give the directions so as to allow as much latitude for individual preferences as possible, but at the same time to call attention to the points which in our opinion must be adhered to.

*Color Comparison.*—In this investigation aqueous solutions of the indicators dissolved with NaOH according to the procedure given by Clark (32) were used. The indicator solutions, as we

used them, were made up in 0.1 per cent concentrations. 5 drops added to 10 cc. of the diluted milk (1 to 19) seemed to give satisfactory color gradations. The buffer standards were prepared according to the tables given by Clark and Lubs (26). In our work they were standardized electrometrically. This, however, should not be necessary for ordinary work. 10 cc. of buffer solution should be placed in each tube. The comparison tubes which we used had an internal diameter of 13 mm.; the tubes need not necessarily have this diameter, but they should not be much larger than this because less light will pass through them. The tubes should be of uniform diameter. The same amount of indicator should be added to the standard buffer tubes as is added to the milk. In some cases it might be found more satisfactory for the individual to determine the amount of indicator which when added to the diluted milk seems to him to produce the most sharp color gradations, but in general it is believed that the amount of indicator specified above will be found satisfactory. A greater volume of a more dilute solution of the indicator can be used. The color comparisons should be made in a comparator block which holds three pairs of tubes; the unknown tube should be placed in the center. It is absolutely necessary to use turbidity blanks and the turbidity blanks should be prepared from the sample of milk which is being tested. The comparator block should be held before a strong light. We found an electric light held behind a thin white paper satisfactory.

*Whole and Skim Milk.*—The simplest procedure for diluting the milk and at the same time one which is sufficiently accurate is to introduce 5 cc. of milk, measured with a pipette, into a 100 cc. graduated cylinder; the cylinder is then made up to the 100 cc. mark with distilled water. Thorough mixing is insured by stoppering the cylinder and shaking. 10 cc. of the diluted milk are pipetted into each of three comparison test-tubes, 5 drops of indicator are added to one tube, the tube shaken, and the comparison of colors is made in the comparator. It frequently happens that the indicator added does not show a match of color with the range for the indicator given in Table VI. If this is found to be the case, another tube of diluted milk should be prepared and the appropriate indicator added. This point is important. The pH of the milk before dilution is found by the use of Table VI. If the milk has developed such an acidity that the casein has started to

separate out, the curd should be thoroughly broken up and mixed with the whey before the sample is taken. After the dilution of such milk has been thoroughly shaken, the casein precipitate can be allowed to settle and the color comparison made on the supernatant liquid.

*Heated Milk.*—The same procedure as used for milk described above may be used with milk which has been heated to boiling for a short time without the loss of water.

*Whey.*—The whey is diluted to 20 volumes with distilled water, the procedure being the same as that described for milk. The pH of the whey before dilution is found by the use of Table VI. The correction factors given in this table do not apply if only part of the casein has been precipitated. This difficulty occurs when the rennet action is incomplete, or in the case of natural souring when the milk is not quite near enough to the isoelectric point of the casein. This condition can usually be recognized by the turbidity of the whey.

*Cream.*—Skim milk should be obtained from the cream, the skim milk diluted to 20 volumes, and the hydrogen ion concentration determined, following the same procedure as that given for milk. The pH of the skim milk before dilution is found by the use of Table VI. The cream is assumed to have the same hydrogen ion concentration as the skim milk obtained from it. The skim milk can usually be obtained from cream by separation in a laboratory centrifuge. If the cream has developed such an acidity that a part of the casein is thrown down during the centrifuging, then the correction factors given do not apply, for the liquid obtained is intermediate between milk and whey.

*Powdered Milk.*—Reconstituted milk, having approximately the average solids not fat composition of normal milk, is prepared using distilled water and this reconstituted milk is diluted to 20 volumes, the procedure being the same as that described for milk. The pH of the reconstituted milk is found by the use of Table VI.

*Evaporated Milk.*—Reconstituted milk is prepared from evaporated milk by diluting the evaporated product with distilled water to approximately its original composition before evaporation. This is usually accomplished by the addition of an equal volume of distilled water. The hydrogen ion concentration of the reconstituted milk is determined by diluting it to 20 volumes, according to the procedure described for milk. The pH of the

reconstituted milk is found by the use of Table VI. An alternate procedure is to introduce 5 cc. of the evaporated milk into a 200 cc. graduated cylinder, add about 5 cc. of water to the milk in the cylinder, and then rinse the pipette by drawing the mixture up into it several times and discharging it. The cylinder is made up to the 200 cc. mark and the procedure as described for milk followed. If it is desired to know the hydrogen ion concentration of the evaporated milk before reconstituting, this can be obtained approximately by subtracting 0.13 pH units from the pH value found for the reconstituted product.

*Sweetened Condensed Milk.*—Dilute the milk with distilled water back to the average solids not fat composition of milk before condensing. Determine the hydrogen ion concentration of this reconstituted milk by diluting it to 20 volumes, following the procedure described for milk. The correction factors given in Table VI do not apply in this case. For the normal product the correction factor of 0.85 pH units is subtracted from the value obtained colorimetrically for the dilution to 20 volumes to give the hydrogen ion concentration of the reconstituted product. If it is desired to know the hydrogen ion concentration of the sweetened condensed milk before reconstituting, this can be obtained approximately, by subtracting 0.30 pH units from the pH value found for the reconstituted product.

The method described by Brown (13) in which small glass cells are used for the color comparisons was found to give results of about the same degree of accuracy as the conventional test-tube comparator using standard buffer solutions, provided corrections were made for the dilution of milk. The dilutions should, however, be made exactly to 20 volumes as described here.

Unless one is familiar with the matching of colors, a little practice may be necessary at first to enable the observer to recognize the gradations in color with such turbid solutions.

#### SUMMARY.

A method for the colorimetric determination of the hydrogen ion concentration of milk, whey, cream, powdered milk, evaporated milk, and sweetened condensed milk, using standard buffer solutions, has been described.

This method is based on the application of correction factors for the effect on the hydrogen ion concentration of diluting the

product with 19 volumes of distilled water in order to reduce the turbidity and to make it possible for light to pass through the solution.

This method gives results which are usually correct within 0.1 pH, the average error being about  $\pm 0.06$  pH.

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## SODIUM DEFICIENCY IN A CORN RATION.\*

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Using corn, casein, and cod liver oil as a basal ration experiments have been in progress in this laboratory for 3 years to study the effect of various mineral supplements to this ration on the production of animals in succeeding generations. Calcium deficiency was recognized at the start and this element was always added to the rations. Addition of one salt as calcium chloride never permitted normal growth in the first generation, while calcium chloride and sodium carbonate additions favored normal growth and vigorous animals in the second generation. This indicated clearly the sodium deficiency in this ration composed of 80 per cent corn.

While this work was in progress Olson and St. John (1) pointed out the variation in sodium contents of wheat and increased the nutritive properties of wheat by supplementing with sodium chloride and sodium bicarbonate. Recently Mitchell and Carman (2) have presented experimental evidence to show the value of supplementing a corn ration with sodium chloride. The results reported here confirm what Mitchell observed pertaining to the relation of sodium to the growth of young rats and in addition include observations through gestation, lactation, and growth in the second generation.

The composition of the rations is given in Table I. Calcium chloride where present was added as a solution to the ration. Diet 1 containing no added sodium salts did not support normal growth as shown by the curve in Chart I representative of the growth of the male rats. The curve representing the growth on

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Diet 2 shows the beneficial effect of adding sodium carbonate to the ration and the satisfactory reproductive record is seen by the data in Table II. One of the females in each of the groups lost her first litter, which explains the mortality of the young. This occasionally happens in case of the first litter and after this the writer has observed the same female to rear seven or eight litters on rations similar to the ones employed here. The adequacy of a ration for successful rearing of the young cannot be questioned through failure of a female to rear successfully her first litter. In

TABLE I.  
*Composition of Rations.*

Diet No.	Calcium chloride.	Sodium sulfate.	Sodium carbonate.	Calcium carbonate.	Yellow corn.	Casein.	Cod liver oil.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	2.2				82.0	15.0	1.0
2	2.2		1.0		81.0	15.0	1.0
3	2.2	1.3			81.0	15.0	1.0
4		1.3		2.0	81.0	15.0	1.0

TABLE II.  
*Reproduction Records of the Rats.*

Diet No.	No. of males.	No. of females.	No. of litters.	No. of young.	No. of young left with mother.	No. of young reared.
1	4	5	0	0		
2	3	4	5	32	27	20
3	2	3	4	25	20	16
4	2	3	3	14	14	12

Diet 3 sodium was added as sodium sulfate. This would not alter the base to acid ratio in the diet as would the addition of sodium carbonate. The success obtained with Diet 3 confirms further that the beneficial effects observed through adding sodium carbonate and sodium sulfate are due to the sodium ion. In a previous publication (3) the author reported successful growth on a synthetic ration where sodium was omitted from the salt mixture. This synthetic ration contained 0.07 per cent sodium compared to 0.03 per cent in the corn ration, which explains the difference in growth of the young rats.

The animals on Diet 1 never consumed over 10.0 gm. of the ration a day per animal and after 100 days the average consumption of feed became less. On Diets 2, 3, and 4, the animals consumed as high as 15.0 gm. of ration when they reached 200 to 300 gm. in weight. The average intake per animal was slightly greater with those animals receiving Diet 4. This diet contained no added chlorine salts, but nevertheless appeared to supply the dietary requirements for growth, gestation, and lactation. The average chlorine intake per day per animal was

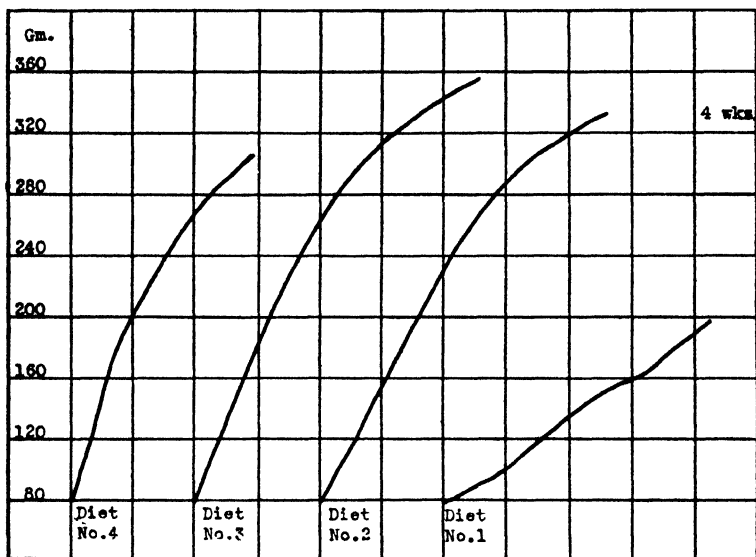


CHART I.

about 5.0 mg. Mitchell (2) suggests from his work that the sodium deficiency of corn is more pronounced than its chlorine deficiency. He found negative chlorine balances with his Ration A which contained no added sodium chloride and was therefore deficient in sodium. In Diet 4 there were no mineral deficiencies except the possible exception of chlorine and this was apparently excluded by the satisfactory animal development. In this work no chlorine balances were determined and it is regretted by the writer that time will not permit them; however, the data presented are sufficient to show the adequacy of the ration in

chlorine for growth, gestation, and lactation. The ability of the animal to conserve its chlorine supply is well known (4, 5). The author has observed this (6, 7) even when a factor directly causing increased chlorine excretion such as high potassium intake is maintained. The writer is inclined to think that the negative chlorine balances obtained by Mitchell and Carman are due to sodium deficiencies which in turn prohibit the animals from functioning normally.

#### CONCLUSIONS.

1. 80 per cent of corn in a ration does not supply sufficient sodium for normal growth. Sodium may be supplied in the form of sodium carbonate or sodium sulfate. Other sodium salts are not excluded.
2. The corn ration fed in these experiments supplied sufficient chlorine for growth, gestation, and lactation.

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## ON THE METABOLISM OF HYDANTOINS AND HYDANTOIC ACIDS.

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Interest in the biochemical behavior of hydantoins originated in studies in which the corresponding open chain compounds—the uramido acids—were obtained from urine. Before methyl hydantoic acid<sup>1</sup> had been synthesized, its presence in the urine as a product of sarcosine metabolism was reported by Schultzen (1). This finding was of simultaneous interest in connection with the formation of uramido acids, and with the course of urea and uric acid formation. The latter two excretory products, according to Schultzen's observations, disappeared on administration of sarcosine. The comparative behavior of urea, carbamic acid, and potassium cyanate, towards sarcosine *in vitro* was studied soon afterwards (2, 3). The disappearance of urea as an excretory product was traced to the interference of unchanged sarcosine with the method used for determining urea (4). Only slight evidence finally remained for the formation of methyl hydantoic acid from sarcosine in the body (Schiffer (5)). Lippich (6) and Dakin (7) point out that another uramido acid or its anhydride thought to be synthesized in the body was probably formed in the procedures used to isolate it.

The hydantoins have, however, been studied in a different connection by Lewis (8). The hydantoin nucleus is present in allantoin, while creatinine and other compounds of biological interest are closely related to it in structure. In a series of papers, Lewis came to the conclusion that the hydantoin nucleus is not attacked in the body.

The experiments with methyl hydantoin here reported were

<sup>1</sup> "Methyl hydantoic acid" designates the  $\beta$ -methyl acid, and "methyl hydantoin" its anhydride, throughout this paper.

undertaken as the result of a suggestion made to one of us some years ago by Professor V. J. Harding, of the University of Toronto, that the possibility of conversion of methyl hydantoin to creatinine by addition of ammonia in the kidney be put to experimental test. Further stimulus was afforded by the observation that methyl hydantoin bore some similarity to blood "creatinine" in the failure of kaolin adsorption, comparative ease of the color reaction with picric acid made alkaline with sodium carbonate, and in the prolonged character of the reaction. Quantitative considerations in the last connection soon disposed of the possibility that any large proportion of blood "creatinine" could be methyl hydantoin, and with the discovery of extensive destruction and oxidation of the latter compound in the body, our interest shifted to the course of this metabolism.

#### EXPERIMENTAL.

##### *1. Procedure and Methods.*

Two dogs were used as experimental animals. They were kept on the experimental diet for some days before the period of observation began. Urines were collected in 24 hour periods, toluene being used as preservative. All periods were terminated by catheterizing and washing the bladder with sterile saline. Total nitrogen was determined by the Kjeldahl-Gunning procedure, ammonia by permutit (9), urea by the method of Van Slyke and Cullen (10), creatinine and creatine by the method of Folin (11), allantoin in one experiment by Wiechowski's (12) method, oxalic acid by the method of Dakin (13), and organic acids in several instances by the method of Van Slyke and Palmer (14). In view of the characteristic reaction of methyl hydantoin with alkaline picrate (15, 16), a simple procedure was adopted for approximate colorimetric determination of methyl hydantoin and methyl hydantoic acid in the urines. A control urine will show nearly the same creatinine value by the Folin method, whether the color development is continued for 10 minutes or 2 hours; a slight fading was the rule in the controls. The same urine with methyl hydantoin added gives greatly increasing values. For example, a control urine in which creatinine was determined by allowing the color to develop before dilution, in separate determinations, for 10 minutes, 1 hour, and 2 hours, gave values for the day of 243, 244, and 239

mg. The same urine with the equivalent of 1.5 gm. of methyl hydantoin per 24 hour sample added, gave values of 322, 737, and 1040 mg. Methyl hydantoin excreted unchanged was therefore taken to be the amount required to be added to control urines of preceding and following days to duplicate the color curve of the urine of the experimental day. If methyl hydantoic acid is present the "hydrolyzed" urine will show the phenomenon of greatly increasing values, when treated in the above manner, and the amount of methyl hydantoin added to hydrolyzed urine of control days to duplicate the color curve of the hydrolyzed urine of experimental days, was taken as "total methyl hydantoin" or methyl hydantoin plus methyl hydantoic acid excreted unchanged. Hydrolyses were carried out with picric acid (11) in all cases. The values at 10 minutes—which appear in the tables as apparent rises in creatinine and creatine—are worthless in determining the amount of methyl hydantoin and methyl hydantoic acid excreted unchanged, because the additional color is a small part of the total, and because fluctuations in creatine excretion interfere. But the large increases above indicated during the 1st and 2nd hour, make the determination easy. Fortunately, also, where methyl hydantoic acid was encountered as a product of methyl hydantoin, the amount was almost equal to that of methyl hydantoin excreted unchanged,—thus making the difference between color curves of unhydrolyzed and hydrolyzed urines of experimental days very large.

Methyl hydantoin and methyl hydantoic acid were prepared from creatinine by the method described in the preceding paper (16). Hydantoic acid was prepared from glycocoll and urea by boiling with baryta as in the method of Baumann and Hoppe-Seyler (2), excepting that separation of the barium salt by use of alcohol was omitted. Barium was removed from the products of hydrolysis by means of sulfuric acid in hot solution and the hydantoic acid allowed to crystallize from the filtrate. Hydantoin was prepared from hydantoic acid by evaporation with hydrochloric acid. All preparations of the four compounds gave entirely satisfactory nitrogen values, and the acids gave correct titration values.

## 2. Metabolism Experiments.

*Experiment 1 (Table I).*—White, short haired female. Weight 10 kilos. The animal was on a diet of 200 gm. of bread and 450 cc. of milk daily, which was consumed quantitatively. On September 27, 1.5 gm. of methyl hydantoin were given subcutaneously in three equal doses, at noon, 5.00 p.m., and 10.30 p.m. The rise in total nitrogen excretion accounts for the nitrogen of the compound given. The compound did not affect ammonia, urea, uric acid, creatinine, creatine, or allantoin excretion. The color curve, with alkaline picrate, of the urine of the experimental day could be duplicated by adding the equivalent of only 15 per cent of the

TABLE I.

*Dog 1.*

Date.	Total N.	Ammonia N.	Urea N (urease).	Creatinine.	Creatine as creatinine.	Allantoin N.	Uric acid N.	Remarks.
	gm.	gm.	gm.	mg.	mg.	gm.	gm.	
1925 Sept. 26	4.10	0.25	3.29	294	133	0.170	0.033	1.5 gm. methyl hydantoin in three doses subcutaneously.
" 27	4.57	0.24	3.43	312	95	0.156	0.033	
" 28	4.00	0.27	3.18	304	123	0.170	0.029	
" 29	4.02	0.22	3.46	312	52	0.169	0.036	

amount of methyl hydantoin given to an aliquot of the control urines.

*Experiment 2 (Table II).*—The same animal was used as in Experiment 1. The diet was changed to 125 gm. of bread and 300 cc. of milk. It was consumed quantitatively. On November 16, 4 gm. of methyl hydantoin were administered subcutaneously in three equal doses. The rise of total nitrogen excretion accounted for all of the nitrogen of the compound. Minor rises in ammonia and urea excretion were noted. The rises in creatinine and creatine excretion are only apparent, and are due to methyl hydantoin and methyl hydantoic acid respectively. The amount of methyl hydantoin excreted unchanged was estimated colorimetrically at 625 mg., or 15.6 per cent of the amount given. The

amount excreted as such and as methyl hydantoic acid was 1175 mg., or 29.4 per cent of that given. An aliquot of the urine was evaporated with hydrochloric acid and the residue extracted with chloroform (16). Evaporation of the chloroform left a crystalline residue, which was recrystallized from absolute alcohol. The crystals melted at 151–155°, and weight for weight gave the same

TABLE II.

*Dog 1.*

Date.	Total N.	Ammonia N.	Urea N (Benedict).	Urea N (urease).	Creatinine.	Creatine as creatinine.	Organic acids.	Remarks.
1925	gm.	gm.	gm.	gm.	mg	mg.	cc. 0.1 N	
Nov. 14			1.88	1.77	223	11	164	
" 15	2.46	0.22	1.88	1.70	220	8	172	
" 16	3.48	0.30	2.17	1.93	271	44	260	4.0 gm. methyl hydantoin in three doses subcutaneously.
" 17	2.47	0.20	1.86	1.76	231	0	154	

TABLE III.

*Dog 1.*

Date.	Total N.	Ammonia N.	Urea N (urease).	Creatinine.	Creatine as creatinine.	Oxalic acid.	Remarks.
1925	gm.	gm.	gm.	mg.	mg.	mg.	
Dec. 1	2.86	0.17	2.03	220	19	5.6	
" 2	2.93	0.12	2.16	228	38	7.8	
" 3	3.70	0.22	2.08	288	69	355.0	4.0 gm. methyl hydantoin in three doses subcutaneously.
" 4	2.72	0.17	1.99	224	26	47.2	

intensity of color with alkaline picrate at 10 minutes,  $\frac{1}{2}$  hour, and 1 hour as known methyl hydantoin. The amount of recrystallized product, calculated to 24 hour volume, was 375 mg. Nearly half of the compound is lost in the amount of alcohol required to remove the pigment and syrupy material, and in any case the amount is much greater than we could isolate from a control



urine after adding only 625 mg. The presence of methyl hydantonic acid above indicated was therefore regarded proven.

*Experiment 3 (Table III).*—The animal and diet were the same as in the previous experiments. 4 gm. of methyl hydantoin were given subcutaneously on December 3. Less than usual was destroyed, the amount excreted as methyl hydantoin being 30 per cent, and that excreted as methyl hydantonic acid 25 per cent of the amount given. The immense amount of oxalic acid found by the method of Dakin (13) suggested the possibility that methyl hydantoin might be oxidized to methyl parabanic acid, which would be decomposed during the alkaline precipitation of the urine in the oxalic acid determination.

*Experiment 4 (Table IV).*—The same animal was used. The diet was unchanged except for daily addition of 2 cc. of concen-

TABLE IV.

Dog 1.

Date.	Total N.	Free oxalic acid.	Total oxalic acid.	Remarks.
1928	gm.	mg.	mg	
Feb. 19	4 28		2.7	
" 20	4 80	144.0	306.0	4.0 gm. methyl hydantoin in three doses subcutaneously.
" 21	3.80	45 0	75.0	

trated HCl, which kept the urine just acid. In addition to the other procedures, the following was used in an attempt to decide whether the oxalic acid found was free or combined. 100 cc. of urine were acidified with 5 cc. of concentrated HCl, evaporated to 25 cc., and continuously extracted with ether for 8 hours. The evaporation with hydrochloric acid might be expected to hydrolyze methyl oxaluric acid (17), but methyl parabanic acid is very stable toward acids. A sample was prepared by the method of boiling theobromine with sulfuric acid and potassium bichromate and extracting with ether. The acid gives no precipitate on adding calcium chloride to its solution acidified with hydrochloric acid, and neutralizing with potassium acetate. Oxalic acid was precipitated quantitatively under these conditions. And when 10 cc. of 0.02 M parabanic acid solution were made alkaline with 2

cc. of 10 per cent sodium hydroxide and boiled 2 or 3 minutes, the calcium oxalate precipitated after adding calcium chloride solution and acidifying with acetic acid required 3.80 cc. of 0.1 N permanganate (theory 4.0). The ether extracts from urines were therefore evaporated over 10 cc. of water, made up to 50 cc., and filtered. Such filtrates are always acid. 20 cc. portions were treated with calcium chloride and potassium acetate directly, and separate 20 cc. portions were boiled with alkali as above, and,

TABLE V.

*Dog 1.*

Date.	Total N.	Ammonia N.	Urea N (urease).	Creatinine.	Creatine as creatinine.	Oxalic acid.	Organic acids.	Remarks.
1926	gm.	gm.	gm.	mg.	mg.	mg.	cc. 0.1 N	
Jan. 4	2.88	0.21	2.24	234	28	14.5	116	4.15 gm. hydantoic acid. neutralized, in three doses subcutaneously.
" 5	2.76	0.22	2.09	242	29	7.2	115	
" 6	3.76	0.09	2.33	245	18	11.2	376	
" 7	2.70	0.23	1.90	222	16	4.5	109	3.5 gm. hydantoin in three doses subcutaneously.
" 8	2.71	0.22	2.05	244	16	15.4	120	
" 9	3.69	0.39	2.09	284	91	11.6	292	
" 10	2.82	0.15	2.25	228	4	11.9	122	
" 11	2.96	0.18	2.37	220	6	10.4	120	

after addition of calcium chloride, acidified with acetic acid. The values for free and total oxalic acid are given in Table IV.

*Experiment 5 (Table V).*—The same animal was used, but now weighed 12 kilos. The diet was the same as in Experiment 2. Hydantoin and sodium hydantoate were used in this experiment. The unchanged hydantoin can be colorimetrically estimated by the same method used for methyl hydantoin. On January 6 4.15 gm. of hydantoic acid were given subcutaneously, after neutralizing with sodium hydroxide. There was a fall in ammonia and rise in urea nitrogen, which may both have been related to increased amount of available base due to the administration of

sodium salt. The rise in organic acids is 260 cc. 0.1 N per day. On adding 0.415 gm. of the acid to one-tenth the urine of January 8, and repeating the determination, the organic acid figure changed from 120 cc. 0.1 N to 452 cc. 0.1 N, —a rise of 332 cc. 0.1 N (theory 350 cc.). On January 9, 3.50 gm. of hydantoin were given subcutaneously in three doses. As in the case of sodium hydantoate the total nitrogen excreted accounted for all of the compound given. There was a rise in ammonia. The color curve of the urine with alkaline picrate could be duplicated by adding the equivalent of only half the hydantoin administered to a urine of a control day, and organic acid showed a rise which would account for the other half of the compound as hydantoic acid. Benzal hydantoin (8, 18), isolated from the urine of January 6 after evaporation with strong HCl, amounted, after recrystallization from alcohol, to 1.34 gm. from half of the day's urine, or the equivalent of 40.9 per cent of the hydantoic acid given (N, 14.81 per cent; m.p. 217.5–218.5°). From half of the urine of January 9, after evaporation with HCl, there were obtained 1.32 gm. of recrystallized benzal hydantoin, or the equivalent of 40.1 per cent of the hydantoin given (N, 14.82 per cent; m.p. 217.5–219°). Wheeler and Hoffman (18) obtained 70 to 80 per cent yields, apparently not recrystallized, when working with pure hydantoin in amounts five times as great. The above isolation would therefore scarcely be expected had the hydantoin not excreted unchanged been excreted as any compound other than hydantoic acid.

*Experiment 6 (Table VI).*—A different animal was used in this experiment,—an adult bitch, weighing 11 kilos. The diet was the same as in Experiment 2, excepting that phosphoric acid and acid phosphates were added to the diet to keep the urine acid. The animal was pregnant and gave birth to a litter of six healthy puppies 3 weeks after the close of the experiment.

On May 28, 4.65 gm. of methyl hydantoic acid, neutralized with sodium hydroxide, were given subcutaneously in three doses. There was a decided rise in urea nitrogen, but the rise in total nitrogen exceeded that of the compound given (0.98 gm.) by 0.35 gm., taking the average of the preceding and following day as control. Urea nitrogen, however, rose 0.70 gm. On June 2, 4 gm. of methyl hydantoin were given, but the last dose was not administered until 11.40 p.m., so that the excretion carries over into the

following day's urine, which began at 9 a.m. The rise in total nitrogen for June 2 and 3 is 0.29 gm. more than the nitrogen of the compound, and the rise in urea nitrogen is 0.66 gm. The amount of methyl hydantoic acid excreted unchanged on May

TABLE VI.  
*Dog 2.*

Date.	Total N.	Ammonia N.	Urea N (urease).	Creatinine.	Creatine.	Free oxalic acid.	Total oxalic acid.	Organic acids.	pH	Remarks.
1926	gm.	gm.	gm.	mg.	mg.	mg.	mg.	cc. 0.1 N		
May 26	3.79	0.24	2.97	284	16	16.3	18.5		5.9	
" 27	3.65	0.33	2.65	280	5	11.2	12.3		6.3	
+ " 28	4.85	0.22	3.33	280	225	84.5	146.0		5.8	4.65 gm. methyl hydantoic acid, neutralized, in three doses subcutaneously.
" 29	3.40	0.23	2.61	288	10	32.6	50.0		6.1	
" 30										
" 31										
June 1	3.14	0.32	2.15	264	28	10.7	14.1		7.0	
" 2	4.00	0.36	2.42	283	21	121.0	184.0		7.0	4.0 gm. methyl hydantoin in three doses subcutaneously.
" 3	3.53	0.37	2.56	264	10	70.5	129.5		6.8	
" 4	3.12	0.34	2.16	245	8	19.7	27.6		7.0	
" 5										
" 6	2.64	0.29	1.84	222	8	15.2	20.2	96	6.7	
" 7	3.65	0.20	2.16	298		20.2	29.2	300	5.8	3.5 gm. hydantoin in three doses subcutaneously.
" 8	2.62	0.24	1.92	228		16.9	24.2	124	6.0	

28 was estimated colorimetrically as 56 per cent of that given. Isolation as methyl hydantoin was accomplished by evaporating the urine with hydrochloric acid, continuously extracting with chloroform, evaporating the chloroform, and recrystallizing the residue from alcohol. The recrystallized product was still slightly

brown. Nitrogen content, 23.9 per cent (theory 24.6). M.p. 152–155.5°, uncorrected (accepted 156°, corrected). The solubilities and color reaction were those of methyl hydantoin. The yield, of *recrystallized* product, calculated to 24 hours, was 0.985 gm. (as methyl hydantoin) or 24.6 per cent of the acid given. The color curve of the unhydrolyzed urine with alkaline picrate showed a very slight rise during the 1st and 2nd hour, indicating a possible trace of methyl hydantoin. The amount of methyl hydantoin excreted unchanged on June 2 and 3 was estimated colorimetrically as 24 per cent of that given, and the "total methyl hydantoin" as 32 per cent. On June 7, 3.5 gm. of unsubstituted hydantoin were given. The results were identical with those in Experiment 5. Colorimetric determination indicated excretion of slightly less than one-half of the compound unchanged, and the rise in organic acids would account for a little more than half of the compound as hydantoic acid. Isolation of benzal hydantoin by a slightly modified method, from half of the urine, yielded 1.81 gm., or 55 per cent of theory (m.p., 217.5°; N, 14.5 per cent). By the same method the isolation of benzal hydantoin from half of a 24 hour urine to which half the amount of hydantoin administered was added, amounted to 67 per cent of the theoretical.

The marked decrease in total nitrogen on the constant diet was chiefly an adaptation to low protein intake, perhaps accentuated by pregnancy. The animal also shows the known decrease in creatinine as the result of cage life to a remarkable degree. The blood urea nitrogen amounted to 14.3 mg. per 100 cc., and "creatinine" 1.5 mg. per 100 cc. at the close of the experiment.

*Experiment 7 (Table VII.)*—Dog 2 was used again in this experiment, after it had weaned its puppies. The daily ration consisted of 150 gm. of a mixture prepared by grinding together 800 gm. of soda crackers and 700 gm. of round steak. 30 cc. of a normal solution of acid sodium phosphate were added to each day's ration. The oxidation of methyl hydantoin in this as in the preceding experiment is smaller than in the case of Dog 1. Urea determinations by the Benedict method, as in Experiment 2, Table II, account for little more, if any, of the extra nitrogen on experimental days than determinations by urease. Methyl urea added to control urines appeared almost quantitatively as a difference between urea determinations by these two methods.

Methyl amine would also appear in this way, since the ammonia determinations subtracted from the Benedict urea determination were done by means of permutit and Nessler's reagent, while the urea plus ammonia nitrogen in the Benedict method is obtained by distillation and titration. While methyl amine is removed by permutit (19) we find that it gives a color entirely comparable with that of ammonia, but only a fifth as great per equivalent of nitrogen. Ethyl amine gives a color one-third as great as that of ammonia, while dimethyl, trimethyl, diethyl, and propyl amines give no color nor yield precipitates. Erdmann (20), no doubt using

TABLE VII.

*Dog 2.*

Date.	Total N.	Ammonia N.	Urea N (Benedict).	Urea N (urease).	Free oxalic acid.	Total oxalic acid.	pH	Remarks.
1928	gm.	gm.	gm.	gm.	mg.	mg.		
Aug. 16	3.02	0.16	2.37	2.34	14.8	19.9	6.6	
" 17	3.87	0.13	2.44	2.31	128.5	193.0	6.3	4.0 gm. methyl hydantoin in three doses subcutaneously.
" 18	3.08	0.12	2.48	2.40	50.7	74.3	6.6	
" 19	2.95	0.09	2.39	2.28	15.2	17.4	6.4	
" 20	4.01	0.09	2.60	2.40	102.3	152.0	6.5	4.65 gm. methyl hydantonic acid, neutralized, in three doses, subcutaneously.
" 21	2.66	0.09	2.09	1.95	10.6	48.8	6.5	

a different Nessler formula, states that amines give lemon-yellow precipitates with the reagent.

Methyl parabanic acid is unaffected in the Benedict urea determination. While it would be decomposed into methyl urea and oxalic acid during the distillation, distillation of methyl urea with the amount of alkali used, not preceded by digestion with potassium acid sulfate and zinc sulfate, yields little nitrogen.

The isolation of the "combined" oxalic acid was attempted. One-half of the urine of each of the experimental days was evaporated to small volume with 12.5 cc. of concentrated HCl, and con-

tinuously extracted with ether. The ether extract was evaporated and sublimed at 135°. Oxidizing acids must be absent in such a sublimation, since unchanged methyl hydantoin otherwise yields methyl parabanic acid. A considerable sublimate of needles was obtained in each case. These sublimates consisted largely of methyl hydantoin. Solutions of the same gave no precipitate with calcium chloride and potassium acetate, but after boiling with alkali, adding calcium chloride, and acidifying with acetic acid, a white precipitate, insoluble in water and titratable with permanganate to a sharp end-point, was obtained. The amount was very small, corresponding to 7 mg. of methyl parabanic acid per day. It was found, however, that of 50 mg. of methyl parabanic acid added to a similar amount of a control urine, only 5.5 mg. were recovered by this procedure. In Experiment 7 only about 70 mg. of methyl parabanic acid could have been excreted per day, hence the result is not surprising.

Although none of the urines showed evidence of cystitis, such as putrefaction or turbidity which would not clear on addition of acid, many of them had been retained 24 hours by the animals. The effect of incubation was therefore studied. Portions equal to one-fourth of the day's urine, from several control days, were incubated with one-fourth the amount of methyl hydantoin given, for 24 hours at 40°C., with and without toluene. The urines were then acidified, concentrated, and extracted as usual. In absence of toluene, the urine became turbid from bacterial growth, and neutral or alkaline. Neither free nor combined oxalic acid could be recovered.

The urine incubated with toluene remained clear and acid, and the values for free and total oxalic acid were those of the original control urine. A previous experiment in which 125 cc. of urine (one-fourth of the daily output) were allowed to stand under toluene at room temperature, after addition of 1 gm. of methyl hydantoin, gave similar results. Colorimetrically determined, the methyl hydantoin fell to about 75 per cent of its original value in several weeks. No increase in oxalic acid could be determined, but much methyl hydantoin could be isolated.

## DISCUSSION.

The chief points of interest in the preceding experiments are the small percentage of methyl hydantoin which is excreted entirely unchanged and the large amounts of free and combined oxalic acid which appear. The latter effect appears much too great to be interpreted as anything but oxidation of the compound given. The failure of such oxidation in the absence of the methyl group is also clear cut.

It is well known that oxalic acid may be destroyed in the body (13), although Wegrzynowski (21) appears to regard the small normal excretion as evidence of very limited formation. The amount of oxalic acid that was excreted in our experiments might only represent a fraction of the extent of actual oxidation of methyl hydantoin. The failure to obtain evidence for the excretion of a great deal of methyl urea (Experiments 2 and 7) does not suggest a much more extensive oxidation than oxalic acid determinations warrant, at least by the two courses of oxidation which we have considered (22). It does not, however, preclude the possibility that the rise in free oxalic acid might be due to decomposition of methyl parabanic acid. The largest amount of total oxalic acid excreted (Table III) was 0.355 gm., which corresponds to 0.45 gm. of methyl hydantoin and 0.11 gm. of nitrogen. The mentioned possibility also is not excluded by the fact that the 24 hour urines were acid, since the urine as secreted might not always be acid.

The two courses of oxidation considered were: (1) transformation of methyl hydantoin to methyl hydantoic acid, with hydrolysis of the latter to methyl urea and glycollic acid, followed by oxidation of glycollic to oxalic acid, and (2) oxidation of methyl hydantoin to methyl parabanic acid. The amount of total oxalic acid excreted on experimental and following days was 30 to 60 per cent greater (Tables VI and VII) when methyl hydantoin was given than when the corresponding amount of methyl hydantoic acid was administered. This may be interpreted as indicating that the acid must be transformed to the hydantoin, previous to oxidation, but is not very conclusive since excretion rates may account for the observation. The only direct evidence for such a course which we have is the observation that urines of August 17 and 20, Table VII, both contained a combined form of oxalic acid which



resisted evaporation with hydrochloric acid, was easily extracted with ether and sublimed. Methyl parabanic acid is known to fulfil these requirements, while one would not expect such behavior of methyl oxaluric acid, or dehydration of the same to methyl parabanic acid.

The nitrogen, of the administered compounds, which in all cases, excepting Table I, equals 0.98 gm., is more completely accounted for in the case of methyl hydantoic acid than in the case of methyl hydantoin. In Experiment 6, Table VI, 56 per cent of the methyl hydantoic acid given was excreted unchanged. The rise in urea nitrogen both in this and the more favorable experiment recorded in Table VII accounts for an additional 12 to 20 per cent. In the case of methyl hydantoin from 30 to 50 per cent was excreted either as such or as methyl hydantoic acid, while irregular rises of ammonia and urea varied from 20 per cent of the nitrogen of the compound to zero. About one-half of the extra nitrogen excreted as the result of methyl hydantoin administration was therefore unidentified.

#### CONCLUSIONS.

1. A considerable portion of methyl hydantoin and methyl hydantoic acid administered to dogs subcutaneously is oxidized, probably to methyl parabanic acid.

2. The oxidation fails in the absence of the methyl group.

3. Both in the case of methyl hydantoin and of unsubstituted hydantoin, a fraction is excreted unchanged, and another fraction as the corresponding acid.

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## STUDIES IN THE DISTRIBUTION OF CHLORIDE AND PROTEIN BETWEEN PLASMA AND SYNOVIAL FLUID.

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Van Slyke, Wu, and McLean (1), 1923, have indicated that the distribution of electrolytes and water between plasma and red blood cells is determined by the Donnan membrane equilibrium. They have also recalculated the data of Loeb, Atchley, and Palmer (2), who studied in detail the equilibrium between plasma and ascitic or pleural fluids in seven instances, and have found a close correspondence between the estimated ratio  $[Cl]_s : [Cl]_f$  and that calculated from the equation derived by Van Slyke, Wu, and McLean for the Donnan membrane equilibrium.

We have applied this equation to the chloride and protein distribution between plasma and non-infected pleural or ascitic fluids in thirty instances with equally satisfactory agreement between the observed and calculated ratios. These results will be reported separately. This paper is concerned with the application of the same equation to the chloride and protein equilibrium between plasma and non-infected synovial effusions. Here again the agreement between observed and calculated  $[Cl]_s : [Cl]_f$  ratio is good in all but two of our nineteen cases. (The experimental data from some of these cases have been included in a separate report (3) dealing with other relationships between plasma and synovial fluids.)

### *Methods.*

Venous blood was obtained with little or no stasis immediately before or immediately after aspiration of the synovial fluid. The

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plasma was promptly separated from the red cells with due precautions to prevent loss of  $\text{CO}_2$  as described elsewhere (3). 3 cc. of plasma or synovial fluid were used for chloride determination by the method of Van Slyke (4), and 5 cc. of plasma or synovial fluid for total nitrogen determination by the Dyer (5) modification of the macro-Kjeldahl method. Non-protein nitrogen was determined, using 2 cc. of plasma or fluid, by the method of

TABLE I.

Case No.	Initials.	Hospital No.	Non-protein N. Mg. per 100 cc. plasma fluid.		Sugar. Mg. per 100 cc. plasma fluid.		Chloride. Mg. per 100 cc. plasma fluid.	
Effusions into knee-joint.								
1	W. P.	271,558	26	23	89	72	354	359
2	O. A.	254,161	23	25	88	82	341	354
3	R. R.	274,498	20	21	130	99	373	385
4	J. A.	275,472	19	22	90	88	347	362
5	A. H.	539,616	23	24	125	86	362	371
6	E. L.	274,137	27	29	116	96	368	381
7	M. G.	273,026	22	19	98	89	367	380
8	G. S.	274,773	25	22	121	110	364	376
9	L. D.	274,134	26	25	118	81	341	356
10	G. L.	275,385	31	31	153	89	345	359
11	W. C.	268,972	30	29	105	101	357	376
12	F. C.	572,511	28	29	102	103	355	375
13	W. L.	274,858	26	23	90	79	354	408
14	K. S.	275,969	24	27	87	76	372	387
15	R. H.	276,006	34	33	151	119	355	366
16	M. G.	250,391	23	30	124	97	368	380
Effusions into prepatellar bursa.								
17	J. D.	580,307	26	27	82	50	367	385
18	L. W.	585,193	33	22	101	69	358	374
19	A. D.	581,973	25	19	99	59	349	370

Folin and Wu (6). Total protein was calculated by subtracting non-protein nitrogen from total nitrogen and multiplying the remaining protein nitrogen by the factor 6.25 to obtain protein. Sugar was determined by the method of Folin and Wu (7), the final dilutions being made in the modified sugar tubes suggested by Rothberg and Evans (8).

Sixteen fluids were obtained from traumatic or chronic non-

infected effusions into the knee-joint, and three from traumatic effusions into the prepatellar bursa.

*Calculations.*

$$r_{sf} = \frac{[BP]_f + \sqrt{[BP]_f^2 + 4[A]_s([A]_s + [BP]_s)}}{2([A]_s + [BP]_s)}$$

TABLE II.

Case No.	Plasma protein. Gm. per liter.	Fluid protein. Gm. per liter.	Estimated plasma H <sub>2</sub> O. Kg. per liter.	Estimated fluid H <sub>2</sub> O. Kg. per liter.	[P] <sub>s</sub> Gm. per kg. H <sub>2</sub> O.	[P] <sub>f</sub> Gm. per kg. H <sub>2</sub> O.	[BP] <sub>s</sub> M.-Eq. per kg. H <sub>2</sub> O.	[BP] <sub>f</sub> M.-Eq. per kg. H <sub>2</sub> O.	[Cl] <sub>s</sub> M.-Eq. per kg. H <sub>2</sub> O.	[Cl] <sub>f</sub> M.-Eq. per kg. H <sub>2</sub> O.
Effusions into knee-joint.										
1	70.1	59.4	0.934	0.937	75.0	63.4	13.3	11.2	106.7	108.0
2	75.4	63.3	0.930	0.939	81.0	67.4	14.3	11.9	103.1	106.2
3	75.1	56.7	0.930	0.945	80.8	60.0	14.3	10.6	111.1	114.8
4	80.1	59.7	0.926	0.942	86.5	63.3	15.3	11.2	105.7	108.4
5	72.9	51.1	0.932	0.949	78.2	53.9	13.8	9.5	109.6	110.1
6	75.9	54.1	0.929	0.947	81.7	57.1	14.5	10.1	111.6	113.1
7	75.8	51.9	0.929	0.949	81.6	54.7	14.4	9.7	111.3	112.8
8	68.4	44.3	0.935	0.955	73.1	46.4	12.9	8.2	109.6	110.7
9	82.5	55.1	0.924	0.946	89.3	58.2	15.8	10.3	103.7	106.0
10	75.2	45.8	0.930	0.953	80.8	48.1	14.3	8.5	104.4	106.1
11	69.7	38.8	0.934	0.959	73.5	40.5	13.0	7.2	107.7	110.2
12	68.9	36.4	0.935	0.961	73.7	37.9	13.1	6.7	106.9	109.5
13	89.3	56.0	0.919	0.945	97.2	59.3	17.2	10.5	108.4	121.7
14	71.8	39.6	0.933	0.958	77.0	41.4	13.6	7.3	112.3	113.9
15	78.9	43.9	0.927	0.955	85.1	46.0	15.1	8.1	107.9	108.0
16	68.6	36.3	0.935	0.961	73.3	37.8	13.0	6.7	111.0	111.4
Effusions into prepatellar bursa.										
17	69.2	46.8	0.935	0.953	74.0	49.1	13.1	8.7	110.6	113.7
18	78.0	48.9	0.928	0.951	84.2	51.4	14.9	9.1	108.9	110.8
19	74.4	35.6	0.930	0.962	80.0	37.0	14.2	6.5	106.8	109.4

(We have used plasma throughout instead of serum, but for convenience have retained the subscript "s" to indicate "plasma.") This is equation (33) from Van Slyke, Wu, and McLean (1). Full discussion of the derivation of this and subsequent equations will be found in the original article. In this equation

$$r_{sf} = \text{calculated distribution ratio for } \frac{[Cl]_s}{[Cl]_f}$$

$[BP]_f$  = m. -eq. per kilo of  $H_2O$ , base bound by protein in synovial fluid.

$[BP]_s$  = m. -eq. per kilo of  $H_2O$ , base bound by protein in plasma.

$[A]_s = [Cl]_s$  = " " " " " chloride in serum.

$[Cl]_f$  = " " " " " " " synovial fluid.

The gm. of water per liter of plasma or fluid were estimated to be 990 - 0.8 P, where P represents gm. of protein per liter. Base

TABLE III.  
*Estimated  $[Cl]_s:[Cl]_f$  Ratio Compared to Calculated  $r_{sf}$ .*

Case No.	Estimated $[Cl]_s:[Cl]_f$	Calculated $r_{sf}$
Effusions into knee-joint.		
1	0.98	0.99
2	0.97	0.99
3	0.97	0.98
4	0.98	0.98
5	0.99	0.98
6	0.99	0.98
7	0.98	0.98
8	0.99	0.98
9	0.98	0.98
10	0.98	0.97
11	0.98	0.97
12	0.98	0.97
13	0.98	0.97
14	0.99	0.97
15	1.00	0.97
16	1.00	0.98
Effusions into prepatellar bursa.		
17	0.97	0.98
18	0.98	0.98
19	0.98	0.97

bound by protein for plasma or fluid was calculated from the equation  $[BP] = 0.177 [P]$  where  $[BP]$  represents milli-equivalents of base bound by protein per kilo of plasma water or synovial fluid water.

### Results.

Table I shows the experimental data on which the calculations found in Tables II and III are based. In Table III is compared

the ratio  $\frac{[Cl]_s}{[Cl]_f}$  estimated from the observed data, and the Donnan  $r, f$ , calculated from the equation derived by Van Slyke, Wu, and McLean. It will be seen that with the exception of Cases 13 and 15 the degree of agreement is as close as that found by Van Slyke, Wu, and McLean for the data of Loeb, Palmer, and Atchley.

#### DISCUSSION.

Loeb, Atchley, and Palmer (2) concluded that a simple membrane equilibrium exists between plasma and ascitic or pleural fluids, influenced in part by the proteins present. Van Slyke, Wu, and McLean (1) believe that this conclusion finds support in the close agreement between the estimated and calculated distribution ratios for Cl,  $HCO_3$  (arterial), and Na, obtained by the use of equation (33).

Several objections may be raised to this equation derived by Van Slyke, Wu, and McLean. Those relating to the estimation of base bound by protein [BP] have been adequately discussed by Peters, Bulger, Eisenman, and Lee (9) and appear to be particularly pertinent where two fluids are compared in which variations in the albumen : globulin ratio probably occur.

The estimation of water content from the protein per liter probably gives a close approximation of the total water content of the plasma or fluid. If there is any appreciable water bound by protein, however, an error would be introduced, since it would appear that the concentration of substances per liter of free water is necessary for a quantitative expression of the Donnan membrane equilibrium.

Variations in protein content of the fluids examined due to the use of potassium oxalate, to variations in  $CO_2$  tension, and to our failure to determine the pH may all introduce appreciable error. We have omitted all cases where the synovial fluid was bacterially infected because of the increased acidity of such fluids (10).

In spite of these objections we feel that our data indicate an interesting analogy in the relationships of synovial, pleural, and peritoneal effusions to plasma; i.e., that in so far as the distribution of chloride and protein is concerned the same type of equilibrium exists between plasma and non-infected peritoneal, pleural, and



synovial effusions; and that the data indicate a simple membrane equilibrium (2).

Until the degree of error introduced by the factors mentioned has been estimated, particularly that in regard to base bound by protein and water bound by protein, we feel that these data should not be accepted as a quantitative demonstration of the Donnan membrane ratio.

#### SUMMARY.

The distribution of chloride and protein between plasma and synovial effusions has been studied in nineteen instances. These data indicate that the equilibrium between plasma and synovial effusion is of the same nature as that between plasma and peritoneal or pleural effusions.

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# THE SIGNIFICANCE OF CYANIC ACID IN THE UREA-UREASE SYSTEM.

## A COLOR TEST FOR CYANIC ACID.

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The close chemical relationship between cyanic acid and urea suggests a corresponding biochemical relationship whenever urea is synthesized or decomposed in animal or vegetable tissues.

For this reason the zymolysis of urea was studied by the writer (1) to see if cyanic acid could be detected among the products of the reaction, which would indicate that the enzyme decomposition of urea proceeds along the same lines as the normal decomposition of urea in solution by acids, alkalies, or heat.

The data obtained led to the conclusion that cyanic acid occurs in the urea-urease system. Mack and Villars (6) also claim to have detected cyanic acid under similar conditions, although they do not ascribe any particular significance to its presence.

Quite recently Sumner (11), working with an extremely active preparation of jack bean urease, has failed to find any indication of the formation of cyanic acid during the zymolysis of urea. Sumner has employed the same analytical technique as the writer, and his results raise the question of the accuracy of the present methods of detecting cyanic acid in complex mixtures, and the validity of the assumption that the cyanic acid comes from the action of the enzyme on the substrate, and not from some other source.

### *Detection of Cyanic Acid in Complex Mixtures.*

Three types of method are available: (1) Conversion of the cyanic acid into urea by incubation with an excess of ammonium chloride; the increase in urea being determined by the xanthidrol method of Fosse (3). (2) Precipitation and separation of the

cyanate as a silver salt. (3) Color reactions of varying degrees of specificity.

The first of these methods is suitable for the detection of cyanic acid in protein solutions free from urease and excess of urea. It has been used by Fosse to demonstrate the formation of cyanates during the oxidation of proteins and amino acids. The method is not applicable to the urea-urease system.

The second method is the most direct. The silver cyanate can be separated from such associated compounds as carbamic acid, carbonic acid, and ammonium bicarbonate, since the silver salt is more resistant to the action of dilute acids. After separation, the cyanate may be converted into ammonia, and estimated.

This method, on account of its simplicity, was applied to the analysis of the urea solutions. There still remains, however, the possibility that other materials associated with the crude enzyme, precipitated by the silver nitrate, may yield ammonia during the subsequent treatment, and thus obscure or mislead the conclusion.

A realization of the time consumed, if not wasted, by the application of inadequate methods to micro analysis made it desirable that the presence of the cyanate, indicated by the silver method, should be confirmed by an independent reaction. The various color tests for cyanic acid were examined to see if any could be applied directly to the urea-urease system. Such a test has not yet been obtained, but a color reaction of considerable delicacy has been devised which will show the presence of cyanate in the silver precipitates obtained from the zymolysis of urea.

Having occasion to test the purity of some specimens of potassium cyanate (prepared by the hydrolysis of urethane), a test for cyanide was made by the Schönbein method.

On addition of about 1 mg. of purified KCNO to 3 ml. of water containing 3 drops of 3 per cent guaiacum tincture and 3 drops of 2 per cent  $\text{CuSO}_4$ , an intense blue color developed similar to the color obtained with pure KCN. The method by which the KCNO had been prepared made it most unlikely that any cyanide was present. Nor did it seem likely that cyanide was formed during the progress of the test, since cyanates are very resistant to reduction.

The specimen of KCNO was found to give a negative response to the Prussian blue test, the thiocyanate test, and the picric acid

test for cyanides as employed by Waller (12). Gottlieb (4), in discussing the toxicity of cyanates, observes that his preparations "gave no positive reaction for cyanide," but omits to mention what tests were employed, nor does he comment on the positive result with the guaiacum reagent.

The delicacy of the reaction between the guaiacum and the cyanate led to a study of the behavior of the cyanate with the other oxidase tests: aloin, benzidine, phenolphthalin, and  $\alpha$ -naphthol, small quantities of a copper salt being added to each test. With phenolphthalin no reaction was observed. With aloin and with  $\alpha$ -naphthol the characteristic oxidation colors were obtained. With benzidine, instead of the usual blue color, a dense sepia purple precipitate was obtained. This soon flocculated and separated from the solution. From this observation the following test was devised.

#### *Color Test for Cyanates.*

To 5 ml. of water add 2 to 5 drops of a 6 per cent alcoholic solution of benzidine, and a couple of drops of a 6 per cent solution of copper acetate. Mix. Add the cyanate solution, which should be neutral or faintly acid. A purple color develops and rapidly changes into a sepia precipitate, which soon separates from the solution.

#### *Notes on the Test.*

1. The test is definitely positive with 1 to 2 drops of a 0.1 per cent KCNO solution (*i.e.* less than 0.1 mg. KCNO), but is capable of a much greater degree of delicacy.
2. Acids interfere with the test by decomposing the compound and hydrolyzing the cyanate. Alkalies inhibit the formation of the colored compound.
3. Cyanides, thiocyanates, thiosulfates, iodides, and bromides interfere with the reaction by forming dense colored precipitates with the benzidine-copper reagent. Sulfates interfere by precipitating the benzidine; for this reason a solution of copper acetate is used.
4. The test is capable of a gravimetric application, as the precipitate can be filtered, washed with water and alcohol, and dried. The compound is sparingly soluble in the commoner neutral or-

ganic solvents. It dissolves with decomposition in pyridine and in acetic acid. It is readily hydrolyzed by dilute mineral acids, the cyanate being converted into the ammonium salt of the acid.

Analysis of the products of the hydrolysis indicates that the compound consists of equimolecular proportions of copper and benzidine with 2 molecules of cyanic acid, which corresponds to the formula:  $\text{Cu}(\text{OCN})_2\text{Bz}$ , and belongs to the same class of body as the benzidine iodide compound recently isolated by Spacu (9). The test also resembles the cyanide reaction described by Pertusi and Gastaldi (8). However, the color given by the cyanate is quite distinct from the dark blue given by most of the other reactants, such as this cyanate (2).

By means of the above test it was found possible to detect small quantities of cyanate in presence of urea and ammonium carbonate, and also in presence of albumin. But on applying the test to the urea-urease system at the early, middle, and later stages of the zymolysis no positive result could be obtained.

Addition of small quantities of cyanate to the carefully neutralized mixture could not be recognized, hence it was concluded that something was present which interfered with the test. This was found to be the enzyme preparation employed. Comparatively large amounts of cyanate can be added to the soy urease solution before a positive response can be obtained to the cyanate test. The urease is precipitated by, and removes, copper from the mixture, but this is not sufficient to inhibit the test, which will appear if sufficient cyanate be added. Addition of more of the urease solution masks the added cyanate.

The benzidine test is not given by silver cyanate, although a faint color will appear after some time. If, however, the mixture of benzidine, copper, and silver cyanate be carefully acidified with  $\text{N}/10$   $\text{HCl}$  the liberated cyanic acid will react to produce the color and precipitate. Excess of acid discharges the color, which returns on neutralization unless the acid has been sufficiently strong to destroy the cyanate.

When the benzidine test is applied to the precipitate obtained by the addition of excess of  $\text{N}/1$   $\text{AgNO}_3$  to a  $\text{M}/10$  urea solution, after 10 hours incubation at  $12^\circ\text{C}$ . with 0.1 per cent Dunning urease, a positive cyanate reaction is obtained on careful neutralization of the mixture. This evidence, although it is indirect,

supports the belief that the precipitate obtained by the action of silver nitrate on the urea-urease system contains a cyanate.

*Origin of Cyanic Acid in Urea-Urease System.*

The cyanic acid detected by the previous methods may arise from four sources: (a) An impurity in the urease preparation. (b) A product of the action of the alkali or the urea on the urease. (c) The zymolysis of the urea. (d) The spontaneous dissociation of urea in aqueous solution. The last of these need not be considered further. It can be clearly proved by means of control experiments that the urea undergoes no measurable spontaneous dissociation in solution under the time and temperature conditions of the experiments.

The possibility of the cyanate arising from the urease preparation is indicated by Sumner's results with the purer preparations. It was on account of this possible source of the cyanate that some of the writer's earlier experiments were carried out with the enzyme solutions enclosed in collodion sacs (1). This method was not found satisfactory. In some experiments the activity of the enzyme decreased considerably; whether owing to loss of a coenzyme or a promoter, or owing to the effect of the membrane, was not investigated. In other experiments the enzyme gradually escaped through a sac made permeable by the action of heat and ammonium carbonate. The later series of experiments, in which the silver nitrate was added directly to the enzyme, gave much more consistent results. The fact that control solutions of urease after inactivation by boiling give no cyanate reaction on incubation with urea is not necessarily decisive since the cyanate or its precursor might be hydrolyzed during the boiling.

*Note on Purification of Soy Urease.*

To aid in the location of the cyanate, a certain amount of work was done on the purification of the soy meal. This work was discontinued on the appearance of Sumner's paper describing the preparation of the much more active jack bean urease (10). However, the following notes may be of interest to workers with soy urease.

The finely ground soy meal was repeatedly extracted with

boiling ethyl ether, or with boiling light petroleum (petroleum ether, b.p. 40–60°C.), in an improvised Soxhlet extractor. The defatted meal was then, in some experiments, thoroughly extracted with alcohol. Incidentally, none of the various alcoholic extracts contained urea or yielded urea on incubation with excess of ammonium carbonate. The almost pure white product obtained by these methods was then freed from excess of protein by a process based on the work of Marshall. The meal was made up in 5 to 10 per cent suspensions and incubated for some hours at 40°C. to favor dispersion. The suspension, freed from the coarser particles, was then acidified by the addition of 20 per cent acetic acid in the proportion of 6 drops of the acid to 100 ml. of the 5 per cent suspension. This caused the formation of a dense curdy precipitate, which gradually subsided. The pale yellow supernatant liquid containing most of the enzyme was filtered off.

While this filtrate may be fractionated in various ways, such as by the addition of  $N/10$  HCl, it is difficult to retain the active urealytic factor. The most successful results were obtained by the use of a 0.5 per cent aqueous solution of safranine (Grübler), which gives a bulky precipitate containing all the urease in the original solution. This was centrifuged, washed, freed from excess of the dye by repeated washings in alcohol, and then decomposed by  $N/10$  HCl, the liberated safranine being removed by extraction.

The safranine precipitate was inactive towards urea, but on being decomposed the urease was liberated.

Safranine was employed on account of the observation by Marston (7) that the azin and the azonium bases precipitate the proteoclastic enzymes, presumably owing to combination between the enzyme and the nitrogen of the diketopiperazine ring.

It may be questioned if the method is as specific as Marston appears to believe, since safranine gives a precipitate with other protein preparations, such as egg albumin, so that the removal of the urease from the solution may be a general adsorption effect and not a specific reaction. Where an enzyme is associated with a protein "carrier," any reagent capable of precipitating the carrier is likely to precipitate the enzyme along with it. For this reason, it would be interesting to know if the azin dyestuffs will

give precipitates with the purified urease preparations obtained by Sumner. The insoluble urease which he has prepared owes its insolubility, one presumes, to the removal of the appropriate colloid carrier.

*Cyanic Acid as the Possible Intermediate in Urea-Urease System.*

Both Kay (5) and Sumner (11) have objected to the hypothesis that cyanic acid is the intermediate product in the zymolysis of urea because the stability of cyanic acid in presence of ammonia is such that the enzyme decomposition of urea could not proceed at its normal velocity. This objection the writer fully realizes. If cyanic acid be the true intermediate product in the urea-urease system, some additional mechanism must be present to bring about its rapid hydrolysis.

Urease by itself appears to have no hydrolytic action on potassium cyanate. The behavior of the free acid in presence of the enzyme is not to be elucidated by a simple experiment, since the continuous formation of urea from the action of the isocyanate on ammonia, and the continuous decomposition of urea owing to the action of urease introduce a set of variables not easily controlled.

That soy urease preparations are not without some action on cyanates is shown by the inhibiting effect on the color test.

In his recent experiments, Sumner has employed a preparation of insoluble jack bean urease, which could be filtered off from the substrate before applying the tests for cyanate. The possibility arises here that in removing the urease any cyanate present may have been removed along with the enzyme.

It would be instructive to know if the results of Sumner would be altered were he to add the silver nitrate reagent directly to the system without any previous separation of the enzyme, and, having washed the precipitate free from ammonia, to decompose the precipitate directly without any previous separation from carbonate and carbamate, a process which may lead to the loss of a small but significant amount of cyanate.

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## GLUTATHIONE CONTENT OF NORMAL ANIMALS.

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Previous contributions from this laboratory have clearly demonstrated that glutathione is specifically concerned in the chemical defense of the body against the action of certain poisons (arsenic, copper, gold, cyanide, methylene blue, etc.). We put forward the theory that the toxic action of these substances is due to a primary disturbance of the cellular oxidation-reduction processes, resulting from a chemical interaction of the poison with the glutathione of the cells. With this knowledge as a foundation it is very desirable to explore this subject in every detail in order to throw some light on the chemical side of cellular mechanics under normal and pathological conditions. In our previous work we were hampered by a lack of knowledge of the actual quantities of glutathione available in the body. The figures given by Hopkins (1) in his original paper could not be relied on because of the large and variable losses involved in the chemical isolation of the substance from biological material. The need for a reliable method seemed to be satisfied with the appearance of the paper by Tunnicliffe (2). After subjecting his method to several tests with regard to reliability and accuracy we adopted it for our purpose.

### *Method.*

*Extraction of Tissues.*—The extraction of the finely macerated material with 10 per cent trichloroacetic acid according to Tunnicliffe is very satisfactory, provided that the tissues are rapidly worked up immediately after the death of the animal. If this precaution is not observed losses are apt to occur.

*Titration.*—After many attempts to use starch as an internal

indicator in the iodine titration we arrived at the conclusion that the values thus obtained might sometimes be as much as 50 per cent higher than the figures obtained with sodium nitroprusside as an external indicator. To mention only one example: A rabbit liver was thoroughly hashed and extracted three times in the usual manner with trichloroacetic acid. The combined extracts were divided into six samples of equal volume. Three of these titrated with starch gave an average of 351 mg. of glutathione per 100 gm. of tissue, whereas the other three titrated with sodium nitroprusside gave 225 mg. per 100 gm. We therefore reached the conclusion that starch is not specific enough as an indicator and we adopted sodium nitroprusside.

*Is All Glutathione in Reduced Form?*—A large number of various trichloroacetic acid tissue extracts were titrated with  $N/100$  iodine both before and after reduction with metallic magnesium (100 mg. for an average sample). Any undissolved magnesium was removed by filtration before the titration. The results so obtained clearly indicated that the increase in the titration value resulting from the reduction rarely exceeded more than 5 per cent. The conclusion was therefore justified that nearly all of the glutathione of the extract was present in the reduced (SH) form and for this reason the figures in the following tables will refer, if not stated otherwise, to the preformed SH- glutathione.

*Specificity.*—After completion of our work, a colorimetric method for the estimation of cystine, cysteine, and glutathione by Sullivan (3) appeared. This method is based on the fact that cystine gives a color reaction with naphthoquinone and so does cysteine after reduction, whereas glutathione reacts only after hydrolysis. Through the cooperation of Dr. Sullivan it was found that the trichloroacetic acid tissue extracts, prepared from liver, muscle, brain, and kidney of the rat, gave no test<sup>1</sup> for either cystine or cysteine when tested with the naphthoquinone reagent. If, however, the extracts are first subjected to acid hydrolysis, they then will yield a positive test. This furnishes experimental proof for the contention of Hopkins (4) that tissues do not contain an appreciable amount of either cystine or cysteine. Hence, the principal objection to the iodine titration method,

<sup>1</sup> Less than 25 parts per million.

*i.e.* the interfering presence of cysteine or cystine, has been removed in the case of normal tissues. The only known substances which occur under pathological conditions, and which give a nitroprusside test are acetone and acetoacetic acid in cases of disturbed fat metabolism, and cysteine and cystine in cases of cystinuria. It is obvious that the method cannot be applied under such conditions, without reservations. It is safe, however, to conclude that the method actually estimates the glutathione of normal tissues.

The naphthoquinone test is not yet adapted for the estimation of glutathione in trichloroacetic acid tissue extracts, and no direct comparison therefore can be made with the results obtained by iodine titration.

*Accuracy.*—If care is taken in the various manipulations the method easily yields results with an error not exceeding 5 to 10 per cent.

Most of the work was carried out on our standard inbred colony of rats, kept on a standard diet consisting of corn and wheat meals, dried milk powder, inorganic salt mixture, and cod liver oil. The food was withdrawn from the animals about 18 hours before the tissues were used and during this time they were kept in a room at 29–30°C. The animals were then weighed and bled to death by decapitation. For the analysis of the entire animal the bodies of several animals were run repeatedly through a meat grinding machine until a perfectly uniform pulp was obtained. For the analysis of the embryos a sufficient number of the proper weight was collected and this material was then minced with scissors and ground up with washed quartz sand. When sufficient material was available the composite sample was divided into several aliquot portions which were analyzed separately. The analysis of the individual organs was carried out by rapidly dissecting out the organs after death and combining a sufficient number of them to yield enough material for analysis. The blood was defibrinated and either analyzed as such or separated into cells and serum by centrifugation at high speed.

In order to eliminate as much as possible individual variations in the glutathione content, a very large number of analyses was made on the same type of material and the results were then averaged.

## DISCUSSION OF RESULTS.

It will be seen from the data included in Table I that the glutathione content of the entire animal declines gradually, the decline being most pronounced between the earlier stages of the embryonic period and the age when the animals have reached a body weight of about 25 gm. From there on the drop is rather small. Part of this diminution in the glutathione content of the animal with increasing age may be due to the development of structures (bony skeleton), which do not contain this substance. However, Jackson and Lowrey (5) found for instance that the

TABLE I.  
*Glutathione of Entire Animal (Rat).*

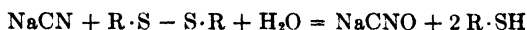
Range of weight of animals.	No. of animals used.	Average glutathione (mg. per 100 gm. of tissue).	Remarks.
<i>gm.</i>			
0.068-0.811	391	60	Embryos.
1.042-1.974	288	58	
2.321-2.948	99	54	
3.895-4.667	104	44	
4.646-4.950	29	36	Newly born.
23-26	38	32	Nursing.
30-50	20	31	Weaned and placed on standard diet plus lettuce.
137-170	20	23	

weight of the ligamentous skeleton of the albino rat never exceeds 18 per cent of the total body weight, whereas the glutathione content drops to nearly one-third of the initial value. Whatever the true explanation may be it is interesting to point out that the glutathione concentration of the body as a whole declines simultaneously with the growth rate. Whether variations in cellular glutathione concentration are directly related to the growth impulse remains to be decided by further work.

As concerns the magnitude of the glutathione content of the whole rat, it is obvious that this animal disposes of considerable quantities of the substance. If the same proportion should also

hold for the human species, an adult weighing 60 kilos would contain about 12 to 14 gm. of glutathione.

The figures in Table I are useful for expressing the relation between the mass of tissue glutathione and that of a fatal dose of a specific poison such as sodium cyanide. 1 mg. of cyanide injected subcutaneously is just sufficient to kill a rat weighing 100 gm. and containing about 25 mg. of glutathione, or, to put it differently, the fatal dose of cyanide is reached when 1 molecule of the poison is injected for each 5 molecules of tissue SH- glutathione.<sup>2</sup> In a recent paper (6) evidence was presented supporting the view that the reaction between cyanide and glutathione is probably best expressed by the following equation:



As we have shown in this paper that most of the glutathione of the tissues is present in the SH form and less than 10 per cent in the S-S form, it is obvious from the above equation that the ratio of 1 mol of NaCN to 5 mols of tissue glutathione agrees very well with a stoichiometric proportion between the two substances and adds further proof that the cyanide action is primarily due to a chemical reaction with the tissue glutathione. Similar considerations might be applied to other specific poisons of glutathione, but this will be reserved for another paper.

Tables II to V inclusive give the variations noted in the glutathione content of various organs at different age periods of the animal. Taken as a whole the liver is the richest organ, next come the kidney, brain and muscle, the latter containing relatively little glutathione. The testes of rats weighing from 142 to 178 gm. gave an average value of 149 mg. per 100 gm. of tissue. These data agree in a general way with those reported by Tunnicliffe (2), but indicate that fluctuations apparently occur in some organs as a result of age.

Finally, Table VI presents the data concerning blood. The first few analyses of rat blood indicated the presence of appreciable amounts of glutathione, which was contrary to Tunnicliffe who claimed that blood does not contain this substance. The glutathione is evidently present in the SH and S-S form in

<sup>2</sup> The molecular weight of SH- glutathione is approximately five times greater than that of NaCN.

TABLE II.  
*Glutathione of Liver (Rat).*

Range of weight of animals.	No. of animals used.	Average glutathione (mg. per 100 gm. of tissue).	Remarks.
<i>gm.</i>			
1.02-1.83	129	151	Embryos.
20-26	108	261	
41-59	135	171	
64-77	30	154	
86-94	33	135	
142-178	45	179	
188-206	20	177	
245-276	30	204	

TABLE III.  
*Glutathione of Skeletal Muscle (Rat).*

Range of weight of animals.	No. of animals used.	Average glutathione (mg. per 100 gm. of tissue).
<i>gm.</i>		
20-26	108	24
41-59	135	25
64-77	30	23
86-94	33	27
142-178	45	32
188-206	20	34
245-276	30	24

TABLE IV.  
*Glutathione of Brain (Rat).*

Range of weight of animals.	No. of animals used.	Average glutathione (mg. per 100 gm. of tissue).
<i>gm.</i>		
20-26	108	102
41-59	135	99
64-77	30	74
86-94	33	78
142-178	45	132
188-206	20	112
245-276	30	40

the blood corpuscles, whereas the serum is free of the substance. These findings agree with the data recently published by Uyei (7) who also found glutathione in the blood cells of several species of animals. The data are in harmony with previous observations (8) showing that blood serum has neither reducing power when

TABLE V.  
*Glutathione of Kidney (Rat).*

Range of weight of animals. <i>gm.</i>	No. of animals used.	Average glutathione (mg. per 100 gm. of tissue).
20-26	108	156
41-59	135	111
64-77	30	52
86-94	33	45 (?)
142-178	45	115
188-206	20	94
245-276	30	19

TABLE VI.  
*Glutathione of Blood of Different Species.*  
(Mg. per 100 Gm. Sample.)

	Defibrinated blood.		Cells.		Serum.	
	SH-gluta-thione.	Total gluta-thione.	SH-gluta-thione.	Total gluta-thione.	SH-gluta-thione.	Total gluta-thione.
Dog.....	20	24	55	79	0	0
Rat.....	22	30	46	53	0	0
Hog.....	31	39	70	93	0	0
Beef.....	35	39	73	81	0	0
Calf.....	36	38	66	74	0	0
Sheep.....	38	41	86	100	0	0
Rabbit.....	49	54	104	114	0	0
Guinea pig....	49	58	143	151	0	0

tested with reduction indicators, nor does it yield a positive nitroprusside test. It was then pointed out that the serum (or plasma) is not concerned with oxidation-reduction phenomena, these being confined to the cells, and this view is again confirmed by the present observations.



We are indebted to Mr. W. T. McClosky for assistance in part of the work.

#### CONCLUSIONS.

1. The reliability of Tunnicliffe's method for the quantitative estimation of glutathione has been established as a suitable method for the analysis of tissues. The method indicates glutathione, as both cysteine and cystine were found to be absent from the tissue extracts.

2. The glutathione is largely present in the SH form, less than 10 per cent occurring in the S-S form.

3. The total glutathione content of the albino rat declines with increasing age.

4. Variations in glutathione content of individual organs occur, due apparently to age.

5. Blood serum does not contain glutathione, but the blood cells do.

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## GLUTATHIONE CONTENT OF TUMOR ANIMALS.

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In the preceding paper (1) we have furnished data concerning the glutathione content of normal animals, particularly albino rats. Those data will serve as a basis for comparison with the data to be presented in the present paper which deals with the glutathione content of the tumors and other organs of albino rats carrying either the Flexner-Jobling carcinoma or a rat sarcoma.

Rats (120 to 180 gm.) of the same strain as the one used in the previous work were inoculated subcutaneously by means of the trocar method with the peripheral (non-necrotic) portion of the tumor. Care was taken in placing the transplants into the side or back regions of the animal in order to avoid interference with the function of vital organs, which might result from pressure by the malignant growth. The sarcoma is a very rapidly growing tumor which reaches enormous size and never produces macroscopic metastases after subcutaneous inoculation. The carcinoma grows much more slowly, and, after having reached considerable size, frequently produces metastatic growths in the axillary lymph glands and other organs.

The animals were fed on the same standard diet as that used in the previous work. The animals were analyzed at various stages of the tumor growth, the food being withdrawn 18 hours previously. The technique of the preparation of the tissue extracts and the analytical procedure were the same as described in the preceding paper. The tumors were dissected out and any macroscopic necrotic portion was carefully removed. The figures in the tables, therefore, refer to the peripheral growing portion of the tumors. After the malignant growth has reached a certain size it is quite impossible to remove every trace of

necrotic material, as the latter infiltrates to some extent the living tissue.

The data in Tables I to IV are averages from a large number of analyses.

#### DISCUSSION OF RESULTS.

Glutathione undoubtedly plays an important part in cellular oxidation-reductions and therefore in the energy exchange of the

TABLE I.

*Glutathione Content of Entire Male and Female Carcinoma Rats.  
(Expressed as Mg. per 100 Gm. of Tissue. One to Four Tumors to Each  
Animal, 53 Animals Used.)*

Average weight of rat minus tumor mass.	Average weight of total tumor mass per animal.	Ratio of tumor weight to body weight.	Normal tissues.		Tumor (peripheral).	
			SH-gluta-thione.	Total gluta-thione.	SH-gluta-thione.	Total gluta-thione.
gm.	gm.	per cent				
105*	Barely palpable tumors.		31	34		
120*	" " "		24	27		
130*	1.1	0.9	24	31	119	149
163	12.7	7.8	16		6	
132*	13.0	9.8	24	31	75	84
167	22.0	12.9	11	12	0	14
146	22.0	15.3	4	5	17	26
157	24.0	15.5	15	18	51	62
155	26.0	17.1	13	17	82	113

\* Female rats, others males.

The various groups of animals were analyzed from 8 to 47 days after inoculation of the tumors.

tissues of higher animals. Hopkins (2) in his original paper describing the isolation of glutathione briefly states "that actively growing cancer cells seem to show a nitroprusside reaction of surprisingly low intensity." This might be taken as evidence that cancer tissue is deficient in glutathione. It is obvious that if this were so it might throw some light on the chemical differences which must exist between normal and malignant tissues. But as Hopkins states, he did not have an opportunity to examine

tumors freshly removed from the body. In making a comparative study of the reducing power of normal and malignant tissues (3), we found some time ago that the living portion of a tumor gives an intense nitroprusside test and reduces methylene blue at least as rapidly under anaerobic conditions as some normal tissues. This would suggest that glutathione is present in considerable amounts.

TABLE II.

*Glutathione Content of Entire Sarcoma Rats.*

*(Expressed as Mg. per 100 Gm. of Tissue. Four Tumors to Each Animal, 50 Animals Used.)*

Average weight of rat minus weight of tumor mass.	Average weight of total tumor mass per animal.	Ratio of tumor weight to body weight.	Normal tissues.		Tumor (peripheral).	
			SH-glutathione.	Total glutathione.	SH-glutathione.	Total glutathione.
<i>gm.</i>	<i>gm.</i>	<i>per cent</i>				
163	3.2	1.9	25	27	137	168
152	3.9	2.5	21	23	159	179
129*	3.6	2.7	27	30	126	148
151	5.4	3.5	13	16	144	176
138*	7.8	5.6	25	26	98	123
144*	22.0	15.3	13	18	108	113
225*	41.0	18.3	10		73	
147	52.0	35.6	10	11	93	97
163*	76.0	46.4	10		29	
153*	89.0	58.5	10		50	

\* Female rats, others males.

The various groups of animals were analyzed from 12 to 32 days after inoculation of the tumor.

The data presented in the tables clearly indicate that as long as the tumor is small (low ratio of tumor weight to body weight), values of about 130 to 180 mg. of glutathione per 100 gm. of tumor tissue are obtained. The few lower figures (Table III) are undoubtedly due to the fact that the necrotic part had not been completely removed. In fact, the above mentioned figures should rather be considered as minimum than maximum values, as it is almost impossible to secure material which is absolutely free of necrotic areas. At any rate, it is evident that the two tumors under consideration contain almost as much glutathione

TABLE III.

*Glutathione Content of Organs and Tumors of Female Carcinoma Rats.  
(One to Four Tumors per Animal, 53 Animals Used.)*

Average weight of rat minus weight of tumor mass.	Average weight of total tumor mass per animal.	Ratio of tumor weight to body weight.	SH- glutathione (mg. per 100 gm. of tissue).				
			Liver.	Skeletal muscle.	Kid- ney.	Brain.	Tumor (periph- eral).
gm.	gm.	per cent					
150	0.7	0.05	180	25	81	104	66
157	0.8	0.50	160	14	0	Trace.	0
136	1.1	0.86	171	18	18	85	85
141	1.2	0.88	161	34	66	126	52
161	2.5	1.6	167	15	81	91	57
157	4.2	2.7	135	14	31	55	40
154	5.2	3.4	171	25	26	42	18
147	8.5	5.8	136	16	43	68	96
146	11.3	7.7	167	25	96	115	117
149	11.8	7.9	162	29	44	87	64
150	12.8	8.5	166	8	25	131	74
145	13.8	9.5	156	16	58	84	29

TABLE IV.

*Glutathione Content of Organs and Tumors of Female Sarcoma Rats.  
(Four Tumors to Each Animal, 15 Animals Used.)*

Average weight of rat minus weight of tumor mass.	Average weight of total tumor mass per animal.	Ratio of tumor weight to body weight.	SH- glutathione (mg. per 100 gm. of tissue).				
			Liver.	Skeletal muscle.	Kid- ney.	Brain.	Tumor (periph- eral).
gm.	gm.	per cent					
134	5.1	3.8	166	23	144	70	129
146	7.4	5.1	145	19	90	75	98
143	9.2	6.4	128	17	80	28	75
156	22.7	14.5	123	4	11	29	65
148	23.1	15.6	133	11	53	54	74
160	29.1	18.1	102	18	0	0	71
171	39.1	22.5	91	8	0	0	71
164	39.5	24.1	89	10	0	0	61

as the normal rat liver, one of the richest organs of the body. The central necrotic portion of the tumor never yields any glutathione, as was ascertained in numerous tests. Tumor extracts,

when tested with Sullivan's naphthoquinone reagent (4) gave a negative test<sup>1</sup> for cysteine and cystine. Hence there is little doubt that the tumors contain glutathione in appreciable amounts and that this substance functions in the same manner in malignant as in normal tissues.

It appears from the data in Tables I and II that tumors, as compared with normal tissues (1), contain a larger part of the total glutathione in the S-S form. It is possible that this may indicate a disturbance in the equilibrium point between the reduced (SH) and oxidized (S-S) form.

The residue of tumor tissue from which the glutathione has been completely removed by repeated extraction yields an intense nitroprusside test. This indicates the presence in tumor tissue of a "fixed" SH group, such as described by Hopkins for normal tissues. This group which is probably attached to a protein is supposed to function in conjunction with glutathione in cellular oxidations.

Our data furthermore reveal an interesting influence which the tumor apparently exerts on the glutathione of the other tissues. It will be noted that as the tumor grows larger in relation to the rest of the body, the so called normal tissues contain less and less glutathione. This diminution is especially pronounced in the case of sarcoma rats, in which some of the organs gave no evidence of the presence of glutathione. It is of course possible that small amounts were actually present, which, however, were too small to be estimated by the analytical method used. Rats inoculated with both types of tumors in the later stages developed marked evidence of malnutrition (cancer cachexia), which was especially pronounced in the case of the rapidly growing sarcoma. The presence of the tumors in no way interfered mechanically with the food consumption or the function of the internal organs, as care was taken to plant the tumors in the proper location. It therefore seems reasonable to conclude that the growing tumor mass gradually causes a disturbance in the metabolism of the other tissues as expressed in a marked diminution of their glutathione content. Whether this effect is primarily due to an excessive demand of the tumor tissue for nutritive material (in-

<sup>1</sup> Less than 25 parts per million.

cluding glutathione) thus depriving the other tissues of their proper share, or whether it is due to a toxic factor, such as the absorption of large masses of necrotic tumor material, cannot be decided at present.

#### CONCLUSIONS.

1. Two types of malignant transplantable mammalian tumors were shown to contain glutathione in amounts comparable to those of the liver, one of the richest organs. The necrotic portion does not contain glutathione.<sup>1</sup>

2. Tumors contain also a so called fixed SH group, which is probably attached to protein.

3. As the tumors grow larger, the glutathione of the rest of the body declines. The significance of this observation to cancer cachexia is pointed out.

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